UNIVERSITY OF ALBERTA

The Role of Ion Channels in the Activation of Murine Macrophages

by

Matthew Albert Rio Lowry

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled The Role of Ion Channels in the Activation of Murine Macrophages submitted by Matthew Albert Rio Lowry in partial fulfillment of the requirements for the degree of Master of Science in Physiology and Cell Biology.

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Dedication

This thesis is dedicated to my mother Princess Veia Atua-Te Matai-Roa-Te Atua-Nui Gwendoline Tinirau Love Lowry whose love and succor helped me through the hardest parts of school and life and whose final words to me bound me to complete this work. For the limitless faith in me and for the shining example of what it meant to be a good and giving person there can never be sufficient thanks or gratitude. You will always be with me mother.

Abstract

The effects of activation with IFN-γ and LPS on the intracellular calcium concentration ($[Ca^{2+}]_i$) and the effects of different potassium channel modulators on the nitric oxide production of the B10-4(S) and P388D.1 macrophage-like cell lines were examined. LPS caused a significant reduction in the $[Ca^{2+}]_i$ of both the B10-4(S) and P388D.1 cells within one hour of treatment that persisted for at least 24 hours. The potassium channel inhibitors tetraethylammonium, 4-aminopyridine, and quinine caused dose-dependent reductions in the nitric oxide production of macrophages, while the potassium channel activator, minoxidol, caused an enhancement of nitric oxide production. The inhibition of nitric oxide production was due to involvement of potassium channels in the priming stage of macrophage activation, since pre-treatment with the priming agent IFN-γ partially protected the nitric oxide response of the macrophages.

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Table of Contents

Chapter 1 : Introduction	1
Origin of macrophages	2
Activation of macrophages	4
Intracellular signaling pathways involved in macrophage activation	6
Involvement of ion channels in macrophage activation	10
Objectives	14
Chapter 2 : The Involvement of Calcium in Macrophage Activation	20
Introduction	20
Materials and Methods	22
Results	26
Discussion	28
Chapter 3 : Involvement of Potassium Channels in the Nitric Oxide Production of Macrophages	42
Introduction	42
Materials and Methods	46
Results	49
Discussion	56
Chapter 4 : General Discussion	79
Future Research	83
Literature Cited	85
Appendix 1 : Validation of the XTT Assay	96
Introduction	96
Materials and Methods	97
Results	100
Discussion	103

List of Tables

Table A1-1. Results of Spearman rank correlation tests between cell number and O.D. 450 nm 107

List of Figures

Figure 1-1. Differentiation of leukocytes from pluripotent stem cells 16

Figure 1-2. Transmembrane structure of ion channels 17

Figure 1-3. Cylindrical structure of ion channels 18

Figure 1-4. Model for the structure of a voltage-gated ion channel 19

Figure 2-1. Loading of P388D.1 cells with fura-2/AM 31

Figure 2-2. Loading of B10-4(S) cells with fura-2/AM 32

Figure 2-3. Ionomycin increases $[Ca^{2+}]_i$ 33

Figure 2-4. Time course of the $[Ca^{2+}]_i$ of a P388D.1 cell treated with ionomycin 34

Figure 2-5. PAF increases $[Ca^{2+}]_i$ 35

Figure 2-6. Time course of the $[Ca^{2+}]_i$ of a P388D.1 cell treated with PAF 36

Figure 2-7. Distribution of the $[Ca^{2+}]_i$ of resting and activated P388D.1 cells 37

Figure 2-8. Distribution of the $[Ca^{2+}]_i$ of resting and activated B10-4(S) cells 38

Figure 2-9. Effects of activators on the $[Ca^{2+}]_i$ of P388D.1 cells after 24 hours 39

Figure 2-10. Effects of activators on the $[Ca^{2+}]_i$ of B10-4(S) cells after 24 hours 40

Figure 2-11. Effects of activators on the $[Ca^{2+}]_i$ of P388D.1 cells after 1 hour 41

Figure 3-1. Calibration of the NO assay with sodium nitrite 64

Figure 3-2. LPS dose response of the B10-4(S) cell line 65

Figure 3-3. LPS dose response of the P388D.1 cell line 66

Figure 3-4. IFN-γ dose response of the B10-4(S) cell line 67

Figure 3-5. IFN-γ dose response of the P388D.1 cell line 68

Figure 3-6. Time course of macrophage NO production 69

Figure 3-7. Cobalt chloride on macrophage NO production 70

Figure 3-8. Tetraethylammonium on macrophage NO production 71

Figure 3-9. 4-aminopyridine on macrophage NO production 72

Figure 3-10. Quinine on macrophage NO production 73

Figure 3-11. Minoxidol on macrophage NO production 74

Figure 3-12. Time course of P388D.1 NO production during ion channel inhibitor treatment 75

Figure 3-13. Time course of B10-4(S) NO production during ion channel inhibitor treatment 76

Figure 3-14. Restoration of inhibitor-reduced NO production by activator pre-treatments (B10-4(S)) 77

Figure 3-15. Restoration of inhibitor-reduced NO production by activator pre-treatments (P388D.1) 78

Figure A1-1. Cell number calibration of the XTT assay for resting B10-4(S) cells 108

Figure A1-2. Cell number calibration of the XTT assay for activated B10-4(S) cells 109

Figure A1-3. Cell number calibration of the XTT assay for resting P388D.1 cells 110

Figure A1-4. Cell number calibration of the XTT assay for activated P388D.1 cells 111

Figure A1-5. Cobalt chloride on B10-4(S) viability 112

Figure A1-6. Cobalt chloride on P388D.1 viability 113

Figure A1-7. Tetraethylammonium on B10-4(S) viability 114

Figure A1-8. Tetraethylammonium on P388D.1 viability 115

Figure A1-9. 4-aminopyridine on B10-4(S) viability 116

Figure A1-10. 4-aminopyridine on P388D.1 viability 117

Figure A1-11. Quinine on B10-4(S) viability 118

Figure A1-12. Quinine on P388D.1 viability 119

Abbreviations

4-aminopyridine	4-AP
Adenosine triphosphate	ATP
Calcium imaging dish	CI dish
Cobalt chloride	$CoCl_2$
Colony stimulating factor	CSF
Diacylglycerol	DAG
Fura-2 acetomethyl ester	Fura-2/AM
Gamma-interferon activating factor	GAF
GTP binding protein	G protein
Guanosine triphosphate	GTP
Interferon	IFN
Interleukin	IL
Intracellular calcium concentration	$[Ca^{2+}]_i$
Inostitol (1, 4, 5)-triphosphate	IP_3
Lipopolysaccharide	LPS
Mitogen activated protein	MAP
Myristoylated alanine-rich C kinase substrate	MARCKS
Nitric oxide	NO
Nuclear factor	NF
Optical density	O.D.
Phospholipase C	PLC
Platelet activating factor	PAF
Protein kinase C	PKC
Tetraethylammonium	TEA
Tumour necrosis factor-α	TNF-α

Chapter 1 : Introduction

Macrophages are phagocytic white blood cells that are essential regulators of acquired immunity and are the main effector cells of non-specific immunity (Abbas *et al.*, 1994). These cells are normally quiescent in the tissues of the body, but become activated for antimicrobial function by interaction with soluble mediators such as cytokines and microbial molecules (Leenen and Campbell, 1993; Abbas *et al.*, 1994; Neumann and Belosevic, 1996). The activation process involves many intracellular signaling complexes including G proteins, intracellular calcium, and protein kinase C (Aderem, 1993; Waga *et al.*, 1993; Reiner, 1994). Experiments using other leukocytes have suggested that ion channels may be involved in the activation processes of macrophages (Schlicter *et al.*, 1986; Eleno *et al.*, 1990; Janiszewski *et al.*, 1992; DeCoursey and Cherny, 1993). This thesis attempts to elucidate a role for ion channels in the intracellular signaling cascades that are responsible for activation of antimicrobial functions in macrophages.

The first chapter is a general introduction to the origin, function, and regulation of macrophages, a review of ion channel structure and function, and a summary of the ion channels found in macrophages.

The second chapter examines the effects of cytotoxic activation on the intracellular calcium concentration of macrophage-like cell lines. The long-term effects (24 hour) and intermediate-term effects (one hour) of incubation with IFN-γ and LPS were examined.

In the third chapter, I examined in-depth the involvement of potassium channels in the nitric oxide production of macrophage-like cell lines by treating the cells with modulators of potassium channels. The time course of the inhibition of nitric oxide production caused by the potassium channel inhibitors was also examined, and inhibitor-treated macrophages were pre-treated with various cytotoxic activators to determine the stage of activation where potassium channels were involved.

The fourth chapter synthesised the results from the various experiments to propose a mechanism by which ion channels may be involved in the intracellular signaling cascades of macrophage activation and propose possible connections between the role calcium channels play in macrophage activation to the role of potassium channels.

In appendix 1, the use of the formazan-based XTT assay to determine reductions in the viability of the macrophage-like cell line was validated by correlating changes in cell number to changes in the optical density at 450 nm of the XTT assay. I also examined the effects of multi-day incubation with potassium channel inhibitors on the cell respiration of the macrophage-like cell lines to confirm that alterations in the cytotoxic function of the cells was not due to reduced cell viability.

Background

Origin of Macrophages

Macrophages are a part of a reticuloendothelial system that extends throughout the circulatory and lymphatic systems, as well as being disbursed through practically every tissue of higher vertebrates (van Furth *et al.*, 1972; Ginsel, 1993). Differentiation of macrophages is a complex process beginning in the bone marrow with haemopoietic stem cells that are responsible for producing all blood cells (Metcalf, 1988). The pluripotent cells possess a virtually unlimited ability to divide to produce daughter cells capable of differentiating along one of several cell lineage pathways (Metcalf, 1988). Subsequent progenitor cells have progressively fewer differentiation pathways they can follow as the cells divide and are influenced by cellular interactions and soluble protein mediators such as growth factors (Leenen and Campbell, 1993). The exact interactions and mediators that the cells encounter, as well as the sequence and timing of those stimuli, induces specific alterations in gene expression, cellular metabolism and morphology to produce one of several types of leukocytes. These include neutrophils, lymphocytes, basophils, eosinophils, megakaryocytes and macrophages (Fig. 1-1).

The differentiation of macrophage progenitor cells involves a number of cytokines, interleukins (IL), and colony stimulating factors (CSF) that are responsible for inducing proliferation and differentiation. A number of these have been cloned including IL-3, IL-2, granulocyte-macrophage-CSF (GM-CSF), macrophage-CSF (M-CSF), IL-4, interferon-α (IFN-α), IFN-β, and IFN-γ (Heyworth *et al.*, 1990; Metcalf, 1991; Leenen and Campbell, 1993). The early stages of macrophage differentiation that are shared with the other leukocyte lineages have yet to be fully characterised. The process is better understood once the progenitor cell's differentiation separates the granulocyte-macrophage lineage from the other lineages. GM-CSF acts on myeloid progenitor cells to produce a population of colony forming cells with the potential to form either macrophages or granulocytes (Metcalf, 1988; Abbas *et al.*, 1994). Interaction with M-CSF causes further differentiation to produce unipotential macrophage colony forming cells (M-CFC) (Metcalf and Burgess, 1982). Thereafter, M-CFCs divide and differentiate to form monoblasts, which are the first bone marrow cells with characteristics typical of macrophages (Ginsel, 1993). The monoblasts are believed to divide only once to produce two promonocytes, each of which also divides only once to produce a total of four bone marrow monocytes (Metcalf, 1988; Ginsel, 1993; Abbas *et al.*, 1994). These monocytes continue differentiating as they enter the circulatory system to distribute throughout the body and complete differentiation within their ultimate tissue location (Metcalf, 1988; Abbas *et al.*, 1994).

Monocytes circulate within the blood and lymph vessels until they encounter the correct stimuli, such as inflammatory soluble mediators or attached proteinaceous markers, to exit the vessels (Ginsel, 1993). Such stimuli cause the blood monocytes to bind to specific ligands on the endothelial lining of the blood vessel wall and migrate between the endothelial and smooth muscle cells into the interstitial spaces in a process called diapedesis (Leenen and Campbell, 1993; Abbas *et al.*, 1994). The monocytes then migrate to their tissue destination and are believed to undergo the final stages of differentiation to become

quiescent resident macrophages with the potential to initiate a number of immune functions (van Furth *et al.*, 1972). This final maturation step is believed to be controlled by cellular interactions and soluble factors present in the individual tissues with the end result being a variety of resident tissue macrophages with specialised functions and unique morphologies. Examples of these macrophages are Kupffer cells (liver), alveolar macrophages (lung), histiocytes (connective tissue), monocytes (blood), osteocytes (bone), interdigitating and dendritic cells (lymph nodes), and Langerhans cells (skin) (van Furth and Sluiter, 1983; Papadimitriou and Ashman, 1989; van Furth, 1989; Leenen and Campbell, 1993). It is believed that many, if not all, macrophages originate as monocytes from the bone marrow. However, there is a growing recognition of the importance of the division of resident macrophages, or a subpopulation of specialised resident macrophages, in tissues as a source of macrophages during non-inflammatory situations (Sawyer *et al.*, 1982; van der Meer *et al.*, 1985; Nakata *et al.*, 1991; Beelen *et al.*, 1994). The exact contribution from such local cell division versus infiltration of blood monocytes under normal homeostatic conditions is uncertain, but it seems likely that both processes occur to maintain the macrophage populations throughout the body.

Activation of Macrophages

Resident macrophages are normally quiescent cells that monitor the local tissue environment. They act as a first line of defence against successful establishment of invading microorganisms or developing tumours because of the many immune, inflammatory, and cytotoxic functions they are capable of initiating or performing (Leenen and Campbell, 1993; Abbas *et al.*, 1994; Beelen *et al.*, 1994). Macrophages phagocytose foreign cells and material, initiate acquired immune responses by presenting processed antigens to lymphocytes in association with major histocompatability complex II molecules, regulate inflammatory responses by production of eicosanoids, regulate other immune responses by production of cytokines, and actively kill foreign cells by production of a

variety of lytic enzymes and cytotoxic molecules (Leenen and Campbell, 1993; Abbas *et al.*, 1994; Neumann and Belosevic, 1996). These functions occur at minimal levels in resident macrophages to conserve metabolic resources and to safeguard the body's tissues. Many of the cytotoxic activities of macrophages are non-selective and damage host tissues as well as destroying foreign cells (Lorsbach *et al.*, 1993; Neumann and Belosevic, 1996). Therefore, the immune functions of macrophages are under tight metabolic regulation, requiring a complex series of stimulations by cytokines or microbial molecules to release the full antimicrobial capabilities of the macrophages.

The activation of macrophages is a two-stage receptor-mediated process (Nathan, 1983; Adams and Hamilton, 1984; Meltzer *et al.*, 1987). In the first stage, a soluble mediator binds to a specific receptor on the surface of the macrophage initiating a series of intracellular signaling cascades that alters gene expression and cellular metabolism in preparation for increased protein and enzyme production and new functions. This is called the 'priming' stage of macrophage activation because it prepares the cell for subsequent full activation. The soluble mediators capable of priming macrophages are often cytokines produced by macrophages or other leukocytes at the site of inflammation or immune response, including GM-CSF, M-CSF, IL-2, IFN-α, IFN-β, IFN-γ, and tumour necrosis factor-α (TNF-α), (Reiner, 1994). TNF-α and IFN-γ are by far the most potent cytokines capable of priming macrophages. The potential for full macrophage function is realised when a second soluble mediator molecule binds to other surface receptors to initiate another series of intracellular signaling cascades. This is called the 'triggering' stage of macrophage activation. While the same cytokines that primed the cell can also trigger the macrophage when applied at higher doses, it is most common that foreign molecules from the invading microorganisms, or developing neoplastic transformants, trigger the macrophages for full immune and cytotoxic functions (Crawford *et al.*, 1994). The exact intracellular signals involved with each stage of macrophage activation have not been

determined and may differ depending on the soluble mediators involved. Nevertheless, numerous intracellular signaling systems have been identified as playing roles in macrophage activation.

Intracellular Signaling Pathways Involved in Macrophage Activation

Cytokines and other soluble mediators of macrophage function use many different methods to transduce activating signals across the plasma membrane of macrophages. However, all begin with the binding of the mediators to specific receptors (Aderem, 1993). One method of signal transduction is the use of single-pass transmembrane receptors with tyrosine kinase activity (Aderem, 1993; Reiner, 1994). The receptor for M-CSF is such an example (Ihle, 1990), and binding of the ligand to the extracellular portion of the receptor induces a conformational change causing autophosphorylation of the receptor/enzyme and activation of the protein tyrosine kinase domain (Aderem, 1993; Hunter, 1993). The receptors for other cytokines, such as IL-2, have no inherent kinase activity but upon binding of the ligand undergo a conformational change to bind with and activate membrane-associated cytoplasmic non-receptor protein tyrosine kinases (PTK) (Samelson and Klausner, 1992; Reiner, 1994). The activated PTK enzymes bind to and phosphorylate other signaling proteins to initiate signaling cascades (Igarishi *et al.*, 1994). Phospholipase Cγ (PLCγ) is activated by PTK-induced covalent modification and hydrolyses phosphatidyl-inositol (4,5)-bisphosphate to produce the second messenger molecules diacylglycerol (DAG) and inositol (1,4,5)-triphosphate (IP_3) (Reiner, 1994; Dekker and Parker, 1994). DAG is an activator of protein kinase C (PKC) enzymes, and IP_3 causes the release of intracellular calcium stores that activates a number of Ca^{2+}-dependent kinases and enzymes, including calmodulin and PKC (Aderem, 1993; Reiner, 1994).

Another method for transducing signals across the plasma membrane is through receptors containing seven-pass transmembrane-spanning domains. Binding of the ligand, such as the chemokine IL-8, causes a conformational change that allows the receptor to

associate with and activate either classical heterotrimeric or small Ras-like guanosine triphosphate-binding proteins (G proteins) (Reiner, 1994; Aderem, 1993). The activated G proteins phosphorylate a number of other proteins and enzymes, including phospholipase Cβ (PLCβ) which again leads to the production of DAG and IP_3 (Nishizuka, 1988; Simon *et al.*, 1991). As mentioned earlier, DAG and IP_3 activate several Ca^{2+}-dependent enzymes, but the enzyme that is affected most directly by these second messenger molecules is protein kinase C.

Macrophages possess several PKC isoforms with different requirements for calcium ion and diacylglycerol (phospholipid) cofactors generated by PLC enzymes (Reiner, 1994). The α, β, and γ isoforms of the conventional PKC enzymes, which are Ca^{2+}-sensitive and phospholipid-activated, have been identified within macrophages (Makowske *et al.*, 1988; Strulovici *et al.*, 1989). Macrophages also possess at least one of the novel PKC isoforms that are phospholipid-activated but Ca^{2+}-independent (the δ isoform) and one of the atypical PKC isoforms whose activities are independent of both Ca^{2+} and phospholipid (the ζ isoform) (Ways *et al.*, 1992; Baier *et al.*, 1993). In general, activation of the PKC isoforms requires binding of the appropriate cofactors, whether phospholipid and/or Ca^{2+}, and phosphorylation of amino acid residues within the enzyme by various kinases (Myers *et al.*, 1985). In addition to causing a conformational change that activates the PKC enzymes, phosphorylation translocates the PKC isoforms from the cytosol to their sites of activity on cellular membranes (Myers *et al.*, 1985).

The PKC isoforms often possess distinctive protein structure around their active sites, giving the PKC isoforms different substrate specificities (Hug and Sarre, 1993). The variety of cofactor requirements of the PKC isoforms may allow the cell to regulate what response occurs, even though the second messenger signals (increased Ca^{2+} and DAG) are common to multiple intracellular signaling cascades (Hug and Sarre, 1993; Strulovici *et al.*, 1990). For example, although PTK receptors and G protein receptors both initiate

elevations in cytosolic Ca^{2+} and liberation of DAG by activation of PLC isoforms, the exact amounts of DAG and Ca^{2+} that is released into the cytosol by the PLC isoforms may vary between the two intracellular signaling cascades. A specific PKC isoform may become activated to a greater extent than the others by the precise levels of Ca^{2+} and DAG present, so that its subset of substrates are preferentially phosphorylated. This could easily push cellular metabolism in specific directions allowing for the observed differences in cellular response when using soluble mediators that bind to different receptors.

The last method for transduction of signals across plasma membranes involves cascades of protein kinases that ultimately activates mitogen activated protein (MAP) kinase. Initiation of the kinase cascades are either dependent or independent of Ras as a receptor (Reiner, 1994). For the Ras-dependent situation, a p^{21Ras} protein acts as a receptor on the surface of the macrophage. Binding of the ligand induces conformational changes that stimulates autophosphorylation of the receptor followed by the sequential phosphorylation and activation of a number of kinases including protein kinase Raf-1, mitogen activated protein ERK kinase and MAP kinase itself (Pelech and Sanghern, 1992; Reiner, 1994). In the Ras-independent cascade, activation of G proteins by association with ligand-bound receptor activates an associated MAP-ERK kinase, thus bypassing some of the Ras-dependent kinases and activating MAP kinase directly (Lange-Carter *et al.*, 1993; Reiner, 1994).

The end result of the activity of these various kinases, whether PKC, MAPK or some other kinase, is the covalent modification of polypeptides at tyrosine, serine or threonine residues to induce conformational alteration of sets of substrates (Krebs and Beavo, 1979; Aderem, 1993). For many of the early cascade kinases, such as G proteins and PTKs, the substrates are themselves kinases or enzymes that then become active to continue the intracellular signaling cascade. However, for the later cascade kinases such as PKC and MAPK, the substrates are proteins that either have direct effects on cellular-

metabolism or are nuclear transcription factors that alter gene expression (Introna *et al.*, 1986; Descoteaux and Matlashewski, 1990; Eilers *et al.*, 1993). An example of a kinase substrate that is directly involved in cellular metabolism is the myristoylated alanine-rich C kinase substrate (MARCKS). MARCKS is upregulated by LPS treatment and activated by PKC-dependent phosphorylation (Aderem *et al.*, 1988; Thelen *et al.*, 1988). The activated form of this protein appears to be essential for phagocytosis by serving as a link between the cytoskeleton and the plasma membrane because of its abilities to bind both myristic acid, which associates with the plasma membrane, and actin (Rosen *et al.*, 1990; Aderem, 1993). There are many examples of nuclear transcription factors that are substrates of kinases, but I will examine only three: the c-fos protein, the gamma-interferon activating factor (GAF), and the NF-κB factor.

Intracellular signaling cascades can control the activity of nuclear regulatory factors in at least three different ways. In the first situation, control exists prior to expression of the nuclear factor. An intracellular signaling cascade through the PKC pathway induces the expression of the c-fos gene following activation of macrophages with LPS (Introna *et al.*, 1986; Radzioch *et al.*, 1987; Higuchi *et al.*, 1988). Once the c-fos protein is expressed, several other gene products are ultimately translated because the c-fos protein is thought to act as a nuclear regulatory third messenger molecule that is capable of transactivating the expression of target functional genes (Descoteaux and Matlashewski, 1990). These gene products are believed to be responsible for altering cellular phenotype and metabolism to produce the activated state in macrophages.

The second method to control the activity of nuclear regulatory factors is by direct covalent modification of a translated factor to induce full activity. The gamma-interferon activation factor (GAF) exists in a latent cytoplasmic form, and it attains an activated DNA-binding form by IFN-γ-dependent protein tyrosine kinases phosphorylating tyrosine residues in its p91 subunit (Shuai *et al.*, 1992). Phosphorylated GAF is believed to be

directly responsible for increasing the expression of several genes associated with IFN-γ induced activation of macrophages (Eilers *et al.*, 1993).

A third way to control the activity of nuclear regulatory factors is by intracellular signaling cascades causing the release of an inhibitory co-factor of a translated protein. The activation of NF-κB occurs within the macrophage cytoplasm when a group of proteins (IκB) dissociates from the hetrodimeric NF-κB because of the activity of an LPS-dependent kinase (Baeuerle and Baltimore, 1988; Ryseck *et al.*, 1992; Ding *et al.*, 1995). The free NF-κB then translocates into the nucleus where it can bind to enhancer or promoter regions containing a decameric consensus motif for NF-κB. This consensus motif is present on a number of LPS-inducible cytotoxic genes, including those for tumour necrosis factor-α, interleukin-1α and β, GM-CSF, M-CSF, IL-8, IFN-β and inducible nitric oxide synthase (Nathan, 1987; Ding *et al.*, 1995; Muller *et al.*, 1993; Grove and Plumb, 1993; Xie *et al.*, 1994). Through the actions of these and other transcription factors, a number of cytotoxic functions essential for the antimicrobial and antitumoural activity of macrophages are ultimately controlled.

Involvement of Ion Channels in Macrophage Activation

The intracellular signaling pathways initiated by cytokines and microbial molecules are diverse including activation of G proteins, production of IP_3 and DAG, induction of calcium cascades, and increased kinase activity (Aderem, 1993; Waga *et al.*, 1993; Reiner, 1994). Experiments in a variety of immune cells indicate a definite, although often elusive, role for ion channels in the activation and cytotoxic function of immune cells. Treating neutrophils with agents, such as potassium channel inhibitors, that induce depolarisation of the membrane potential from the resting value around -60 mV inhibits the ability of neutrophils to produce reactive oxygen molecules (Martin *et al.*, 1988; Krause *et al.*, 1993). Proton channels are important in activating the respiratory burst of neutrophils and macrophages by helping to remove excess H^+ ions produced by the electron transport chain

that forms reactive oxygen intermediates (DeCoursey and Cherny, 1993; Murphy and Forman, 1993). Calcium channels are proposed to play a role in the release of inflammatory mediators that occurs during mast cell degranulation by providing a pathway for the entry of extracellular Ca^{2+}, which is essential for the fusion of granules and plasma membrane (Penner and Neher, 1988; Janiszewski *et al.*, 1992). Potassium channels were also found to play a role in mast cell degranulation, since treating mast cells with inhibitors of potassium channels, such as quinine, 4-aminopyridine, and sparteine, promoted the release of histamine (Eleno *et al.*, 1990; Janiszewski *et al.*, 1992). In natural killer cells, potassium channels play an opposite role in the cell's cytotoxic functions since treatment with the potassium channel inhibitors tetraethylammonium (TEA) or 4-aminopyridine (4-AP) abrogated target-cell killing (Schlicter *et al.*, 1986). Similarly, Haslberger *et al.* (1992) found that treating murine peritoneal macrophages or cells of the RAW 269 murine macrophage cell line with 125 μM of the potassium channel blocker quinine prevented LPS-induced TNF-α release. The results from these experiments suggest that ion channels are involved in the signaling pathways used in the cytotoxic activation of macrophages against microbes.

Ion channels are protein complexes that form pathways through the hydrophobic regions of the plasma membrane and allow specific ions to rapidly pass (Hille, 1992). In many cases, ion channels are normally closed, preventing the passage of molecules into or out of the cell. They open, or gate, in response to various stimuli including changes in the electrical state of the cell (voltage-gated channels), binding of specific molecules to the channel (ligand-gated channels), or covalent modification of a region of the channel (Catterall, 1988; Hille, 1992; Unwin, 1993). When an ion channel opens, the ions flow down their electrochemical gradient that depends both on the relative concentrations of any particular ion between the inside and outside of the cell and on the membrane potential of the cell. Ion channels are integral membrane proteins with multiple transmembrane

segments that weave back and forth between the cytoplasm and the extracellular environment in the form of α-helical rods or random-conformation loops. (Fig. 1-2). The various ion channels are structurally similar. Each channel consists of a number of homologous regions (usually four) that are either domains of a single subunit, as in the sodium and calcium channels, or structurally similar subunits, as in the potassium channels (Fig. 1-2). The subunits or domains spontaneously arrange themselves side by side so that a cylindrical structure is formed through the plasma membrane (Fig. 1-3) (Catterall, 1988; Aidley and Stanfield, 1996). A non-helical hydrophilic transmembrane loop from each subunit or domain lines the pore to provide a hydrophilic continuum between the extracellular environment and the cytoplasm.

Ion channels that open or close in response to alterations in the membrane potential (voltage-gated ion channels) possess a transmembrane α-helical spanning region that contains repeated positive amino acid residues that act collectively as a voltage sensor (Fig. 1-4) (Catterall, 1988; Hille, 1992). Changes in the membrane potential alters the force exerted on the charged transmembrane helix within each subunit and thereby the position of the helix within the lipid bilayer in relation to the rest of the ion channel subunit. The changes in position alter the conformation of the entire ion channel subunit such that the pore is either forced open to allow ions free passage or forced closed. Ligand-gated ion channels are structurally similar to voltage-gated ion channels with homologous regions that form a cylindrical channel, but they often lack the charged transmembrane helix voltage sensor (Unwin, 1993). Instead, a specific ligand binds to a receptor domain and induces a conformational change that gates the ion channel (Unwin, 1993; Aidley and Stanfield, 1996). Alternatively, a closely-associated but separate receptor protein binds the ligand and thereafter interacts with the ion channel to induce a conformational change that opens or closes the pore through the ion channel (Jessel and Kandel, 1993; Unwin, 1993). Some ion channels have domains projecting into the cytoplasm that act as substrates for

regulatory enzymes, such as phosphatases or kinases. The opening and closing of the ion channels may be controlled , either solely or in combination with alterations in membrane potential or binding of specific ligands, by the covalent modifications of these domains (Damjanovich *et al.*, 1992; Jessel and Kandel, 1993).

In addition to domains that control the opening of the gate, ion channels also contain domains that selectively pass ions and that inactivate the channel. The domain(s) involved in selectively allowing the passage of certain ionic species operate through a combination of charged groups and a restriction of the size of the hydrophilic pore (Fig. 1-4) (Catterall, 1992; Hille, 1992). This selectivity filter prevents ions larger than a specific size to pass and repels ions of the same charge as the filter. This means that specific types of ion channels principally carry a single ionic species, but these channels can and do allow other smaller ions of charge similar to the principal species to pass although at much lower conductances. There are also ion channels that do not show selectivity and allow any ion of the correct charge to rapidly pass into or out of the cell (Hille, 1992).

The domain that inactivates the ion channel works by blocking the pore after the channel has been open for a certain amount of time or after a specific physiological event has occurred, such as a phosphorylation of the ion channel's subunits (Hille, 1992). Voltage-gated Na^+ channels will spontaneously inactivate after the ion channel has gated open by a conformational rearrangement that moves a "tethered ball" of amino acids into the pore (Hille, 1992; Jan and Jan, 1992). The ion channels remain inactivated until the cell has repolarized by having the membrane potential return to its resting state. This resets the inactivator's conformation and also closes the gate thereby preparing the channel to respond to the next stimulus (Hille, 1992).

Macrophages possess at least six types of ion channels, including an inwardly rectifying potassium conductance, an outwardly rectifying potassium conductance, a calcium-activated potassium conductance, a chloride conductance, a proton conductance,

and a non-specific cationic conductance (Randriamampita and Trautmann, 1987; Gallin and McKinney, 1988; Nelson *et al*, 1990; Holevinsky *et al.*, 1994). The main function of potassium channels within macrophages is believed to be the maintainenance of the membrane potential between -30 to -56 mV (Gallin, 1991). Chloride channels play a smaller role in maintaining the membrane potential and are also involved in regulating the volume of cells (Hille, 1992). The proton channels of macrophages are believed to be exclusively involved in the extrusion of H^+ ion from the cytoplasm during the respiratory burst of macrophages (Holevinsky *et al.*, 1994). No voltage-gated calcium channels have been detected in the plasma membrane of macrophages (Gallin, 1991), although it is believed that second messenger-gated calcium channels are present in the plasma membranes of various organelles. In addition to the known electrophysiological functions, the ion channels may have additional functions in the metabolism of macrophages that have yet to be determined.

Objectives

Ion channels are well-suited for the propagation of intracellular signaling cascades because they interface so many processes and structures. Ion channels possess extracellular domains that may have ligand-binding properties, they possess intracellular domains that interact with other proteins and that are sites of phosphorylation, and the effects of altering the ion channel's activity can have widespread consequences over the entire cell (Hille, 1992; Catterall, 1992; Unwin, 1993; Wickman and Clapham, 1995). The many experiments indirectly examining the role of ion channels in the cytotoxic functions of leukocytes clearly suggest that an alteration in ion channel activity, particularly calcium channels and potassium channels, affects the function of the leukocyte. The most probable way that modification of ion channel activity could be affecting so many disparate processes is if the ion channels of the various leukocytes were involved in a process common to all.

The work presented in this thesis attempts to more fully explore the involvement of calcium channels and potassium channels in the activation process of macrophages and to relate that involvement to the intracellular signals of activation. The aims of this thesis are to determine if treatment with the cytotoxic activators LPS and IFN-γ would increase the intracellular calcium concentration of macrophages, and to determine if treatment with ion channel inhibitors would decrease the nitric oxide production of LPS/IFN-γ activated macrophages without decreasing cellular viability. Since macrophages do not have voltage-gated calcium channels embedded in their plasma membranes to monitor by voltage patch clamping, the involvement of calcium channels had to be assessed indirectly by examining the changes in intracellular calcium concentration using the calcium-sensitive ratiometric fluorescent dye fura-2/AM. The involvement of potassium channels in the activation of macrophages was examined by determining the effects on nitric oxide production of removing potassium channel activity by treating the macrophages with specific pharmacological blockers of potassium channels.

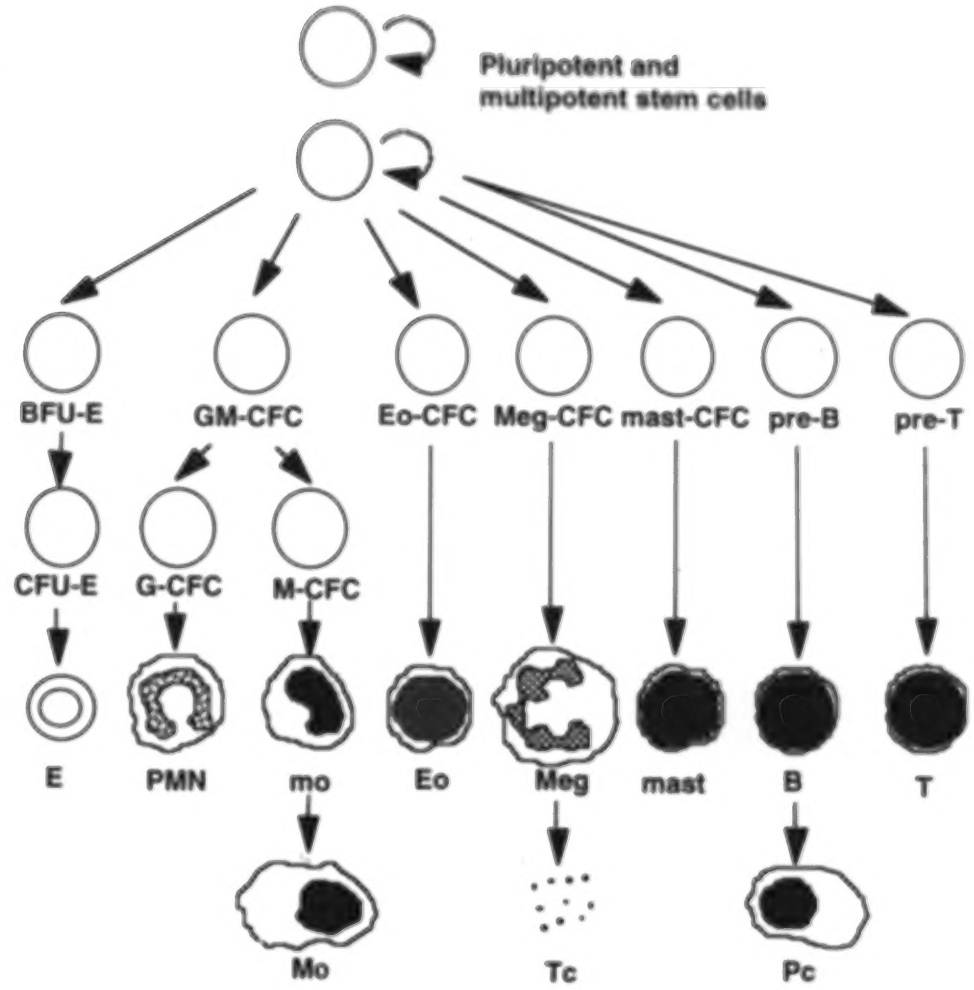

Figure 1-1. Production of leukocytes from pluripotent stem cells. BFU-E, burst-forming unit-erythroid; CFU-E, colony-forming unit-erythroid; E, erythrocyte; GM-CFC, granulocyte-macrophage colony-forming cell; G-CFC, granulocyte CFC; M-CFC, macrophage CFC; PMN, polymorphonuclear granulocyte; mo, monocyte; Mø, macrophage; Eo, eosinophil; Meg, megakaryocyte; Tc, thrombocytes; B, B lymphocytes; T, T lymphocytes; Pc, plasma cell. [Modified from Leenen and Campbell, 1993].

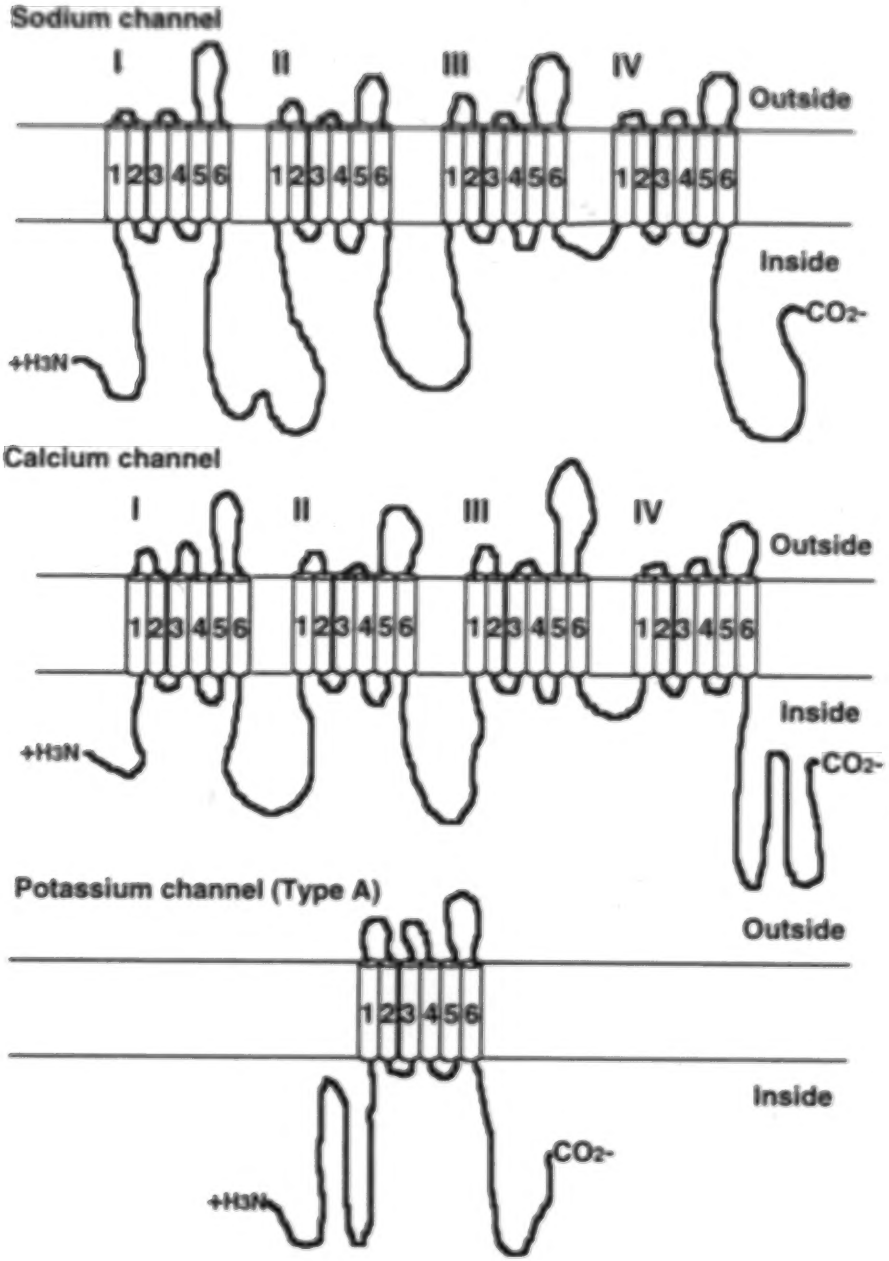

Figure 1-2. Proposed transmembrane structures of the principal subunits of three voltage-gated channels. [Modified from Catterall, 1988].

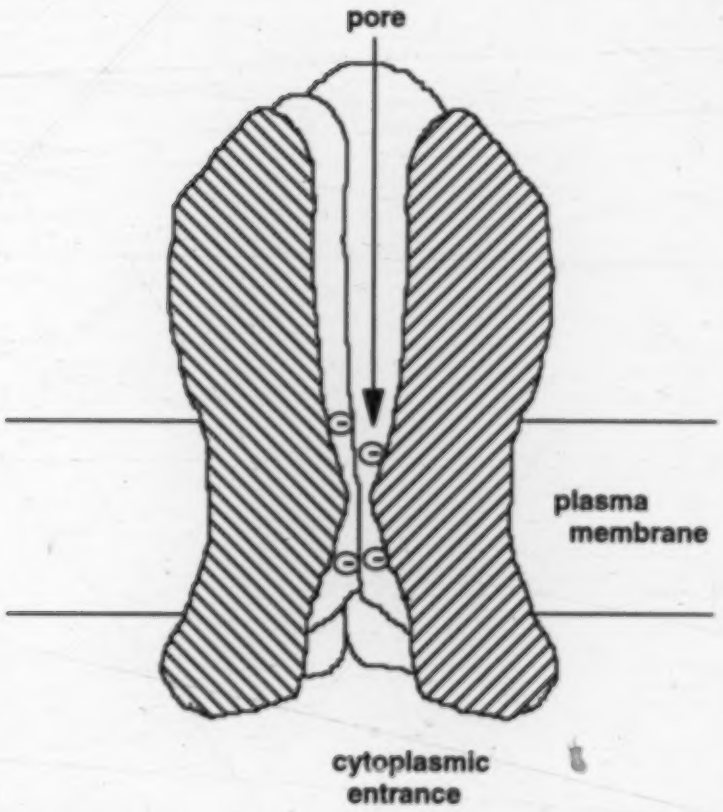

Figure 1-3. Cylindrical structure of ion channels forming a passage that penetrates lipid bilayers. [Modified from Unwin, 1993].

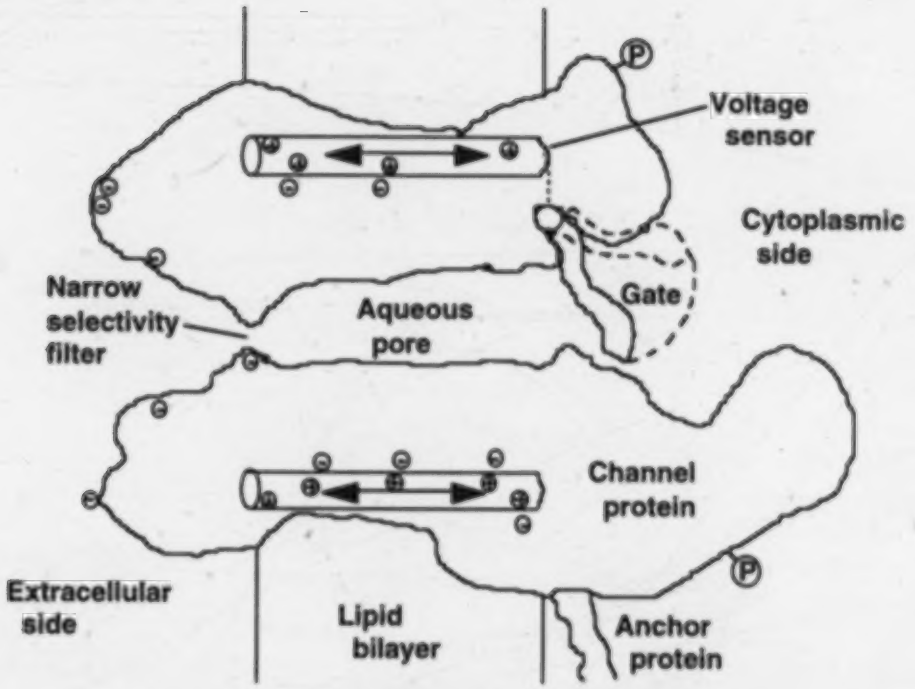

Figure 1-4. Model for the structure of a voltage-gated ion channel. Each subunit contains a positively-charged α-helix that acts as a voltage sensor which opens or closes a gate region depending on the membrane potential of the cell. In addition, the channel contains a selectivity filter that allows only specific ions to pass. [Modified from Hille, 1992].

Chapter 2 : The Involvement of Calcium in Macrophage Activation

Introduction

Calcium plays a well-documented role in the intracellular signaling of many types of cells. The cytosolic concentration of calcium ions ($[Ca^{2+}]_i$) regulates the activity of a number of calcium-dependent processes and enzymes within the cytoplasm of cells, including the kinase activity of protein kinase C (PKC) and the regulatory functions of calmodulin (Scharff and Foder, 1993; Reiner, 1994). In many cell types, changes in the $[Ca^{2+}]_i$ are brought about by the production of inositol 1,4,5-triphosphate (IP_3), and other second messenger molecules, following the binding of regulatory molecules to receptors on the surface of the cell (Aderem, 1993). These second messenger molecules release Ca^{2+} ions from the endoplasmic reticulum, nucleus, and other intracellular stores as well as activating calcium ion channels and ATP-driven Ca^{2+} pumps in the plasma membrane to facilitate the entry of calcium from the extracellular environment (Scharff and Foder, 1993). By regulating the activity of calcium-dependent enzymes and proteins, alterations in the cytoplasmic concentration of Ca^{2+} play an indispensable role in the intracellular signaling cascades that control gene expression and ultimately the metabolism of cells (Aderem, 1993; Scharff and Foder, 1993).

Although transient increases in calcium concentration control some aspects of macrophage metabolism (Scharff and Foder, 1993), the role of calcium transients in the activation of cytotoxic functions within macrophages has been controversial. It is clear that certain inflammatory mediators such as platelet activating factor (PAF) and chemotactic factors induce transient biphasic increases in the $[Ca^{2+}]_i$ of macrophages within minutes of application (Randriamampita and Trautmann, 1989; Katnik and Nelson, 1993). Whereas these factors induce chemotaxis and the production of inflammatory regulators by macrophages, they neither prime nor trigger cytotoxic functions, such as production of

reactive oxygen or nitrogen intermediates (Waga *et al.*, 1993; Abbas *et al.*, 1994).

When researchers initially looked for calcium transients following treatment of the cells with mediators known to fully activate macrophages for cytotoxic functions, such as interferon-γ (IFN-γ) and lipopolysaccharide (LPS), they failed to detect them (Drysdale *et al.*, 1987; Maudsley and Morris, 1987; Letari *et al.*, 1991a). Eventually, researchers found that perfusion with LPS or IFN-γ caused transient increases in the $[Ca^{2+}]_i$ of macrophages loaded with the calcium-sensitive fluorescent dye fura-2/AM within the first few minutes of application (Letari *et al.*, 1991b; Raddassi *et al.*, 1994; Rotnes *et al.*, 1994). However, it was found that these increases were at least partially due to cross-reactivity with the PAF receptor, and that such calcium transients were not important to the activation of macrophages for cytotoxic functions (Waga *et al.*, 1993; Raddassi *et al.*, 1994). Nevertheless, it is clear that calcium plays some role in the priming stage of macrophage activation. Treatment of macrophages with pharmacological agents that alter the intracellular calcium concentration, such as calcium ionophores and inhibitors of endoplasmic reticulum Ca^{2+}-ATPase, primed the macrophages for subsequent full activation (Jun *et al.*, 1996; Park *et al.*, 1996; Raddassi *et al.*, 1994).

Most studies examining the role of calcium in activation looked for immediate effects within the first few minutes of activator treatment, but the effects of activator treatment on the $[Ca^{2+}]_i$ at much longer incubations, on the order of hours, remained unexplored. Activation of macrophages with LPS and IFN-γ induces reactive oxygen intermediate production within a matter of tens of minutes and may be influenced by immediate effects on $[Ca^{2+}]_i$ (Neumann and Belosevic, 1996). However, nitric oxide production is not induced until several hours after activator treatment (Stuehr and Marletta, 1987; Neumann and Belosevic, 1996), and it was hypothesized that a later alteration in $[Ca^{2+}]_i$ may be responsible for the induction of nitric oxide production. To clarify the role of calcium in the activation processes of macrophages, experiments were undertaken to examine the

long-term effects of treatment with IFN-γ and LPS on the $[Ca^{2+}]_i$ of the B10-4(S) and P388D.1 macrophage-like cell lines using the calcium-sensitive ratiometric fluorescent dye fura-2/AM.

Materials and Methods

Reagents

Recombinant murine interferon-γ (IFN-γ) was purchased from Genzyme (Boston, MA) and lipopolysaccharide (LPS) from *Salmonella typhimurium* was obtained from Difco Laboratories (Detroit, MI). Fura-2/AM and Pluronic F-127 were purchased from Molecular Probes Inc. (Eugene, OR).

Cell Culture

Cells of the macrophage-like B10-4(S) and P388D.1 cell lines were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle medium (Gibco-BRL, Grand Island, NY) supplemented with 10 % fetal bovine serum (HyClone Laboratories Inc., Logan, UT) and 50 μg/mL gentamicin (Gibco-BRL, Grand Island, NY) (complete DMEM). The P388D.1 cell line consisted of slower growing cells at a later stage of differentiation, and was obtained from the American Type Culture Collection (Koren *et al.*, 1975). The B10-4(S) cell line consisted of rapidly dividing cells with morphology typical of less mature macrophages, and was a kind gift from Dr. D. Radzioch, McGill University. The B10-4(S) cell line was immortalized from bone-marrow derived macrophages isolated from C57BL/10.A ($B10A.Bcg^S$) mice that were susceptible to Bacillus Calmette Guerin (Radzioch *et al.*, 1991).

Loading Macrophages with Fura-2/AM

Macrophages were suspended to a cell density of 1.0×10^5 cells/mL in complete DMEM, and 400 μL of the suspended cells (4.0×10^4 cells) were seeded into a sterile glass-coverslip bottomed 35 mm plastic calcium imaging dish (CI dish). The cells were

allowed to attach to the CI dish for one hour at 37°C and 5% CO_2, the medium was aspirated off, and the dish was filled with complete DMEM containing 2.5 μM fura-2 acetomethyl ester (fura-2/AM) (loading medium). The fura-2/AM (Molecular Probes Inc., Eugene, OR) had previously been solubilized in 2.5% pluronic-F127 (Molecular Probes Inc.)/dimethyl sulfoxide (DMSO - Caledon, Georgetown, Ont.) and then diluted in complete DMEM to bring the final concentrations to 2.5 μM fura-2/AM, 0.0025% pluronic-F127, and 0.1% DMSO. The addition of pluronic F-127 to the DMSO increased the solubilization of fura-2/AM and resulted in greater dye entry into the cells and a more uniform dye distribution within the cells (Poenie *et al.*, 1986). The macrophages were allowed to load with fura-2/AM for 1 hour at 37°C and 5% CO_2. The loading medium was then discarded, the cells were washed 3 times with 2 mL aliquots of 37°C mouse extracellular bathing solution (MEBS - 4.5 mM KCl, 145 mM NaCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$ and 10 mM HEPES at pH 7.2), 2 mL of MEBS was placed in each culture, and the cultures were maintained at 37°C.

Imaging of Intracellular Calcium

The $[Ca^{2+}]_i$ of the macrophages was estimated using standard dual wavelength ratiometric imaging procedures employing the calcium-sensitive fluorescent dye fura-2 (Tsien *et al.*, 1985; Goldberg *et al.*, 1992). In brief, the 540 nm emissions of fura-2/AM loaded macrophages that were sequentially excited at 340 nm and 380 nm were detected using an intensified charge-coupled device (CCD) camera (Paultek Imaging, Nevada City, CA) attached to a Zeiss Axiovert 135 fluorescent inverted microscope equipped with a 100x Fluar objective lens (Zeiss, Ontario, Canada). Photobleaching and photodamage were minimized by employing neutral density filters in the excitation light path from the 100 watt Hg/Xe-arc lamp and by using short ultraviolet exposure times (van Goor, 1997). The images were digitized, stored and analyzed using the program Ratio 1.3 (kindly provided by Dr. S. B. Kater, University of Utah School of Medicine) on a Macintosh Quadra 950

computer equipped with a Quickcapture frame grabber board (Data Translation, Ontario, Canada). While being examined, the dishes were maintained at near physiological temperature (36-38°C) using a petri-dish warmer.

Calibration of the Calcium Imaging Program

Ratio 1.3 approximated the calcium concentration of a selected rectangle by calculating the calcium concentration of each pixel within the selected area using equation 1 and then averaging the calculated concentrations (Grynkiewich *et al.*, 1985; Van Goor, 1997).

[1] $$[Ca^{2+}] = (K_d \times Q) \frac{(R - R_{min})}{(R_{max} - R)}$$

where: $[Ca^{2+}]$ is the intracellular calcium concentration, Q is the ratio of 380 nm-induced fluorescence with no calcium (F_{min}) divided by the 380 nm-induced fluorescence with saturating amounts of calcium (F_{max}), K_d is the apparent Ca^{2+} dissociation constant for fura-2, R is the ratio of the observed 340 nm-induced fluorescence divided by the observed 380 nm-induced fluorescence, R_{min} is the ratio of the 340 nm-induced fluorescence/380 nm-induced fluorescence obtained with no calcium, and R_{max} is the ratio of the 340 nm-induced fluorescence/380 nm-induced fluorescence obtained with saturating calcium concentrations.

The calcium imaging system was calibrated at 20 °C to determine the K_d of fura-2, the R_{min}, the R_{max}, and the relation F_{min}/F_{max}. The fluorescence signals following sequential excitation at 340 nm and 380 nm of fura-2 pentapotassium salt (100 µM) in cell-free solutions of known Ca^{2+} concentration entrapped in glass capillary tubes with a path length of 20 µm (Vitro Dynamics, Rockaway, NJ) were determined using the 100x Fluar objective lens (Van Goor, 1997; Christopher, 1997). Calibration solutions from 0 to 39.8 µM free Ca^{2+} were obtained from a Ca^{2+} Calibration Buffer Kit with magnesium II (Molecular Probes Inc., Eugene, OR). The ionic composition of the calibration solutions included: zero to 10 mM CaEGTA, 1 mM free Mg^{2+}, 100 mM KCl, and 10 mM MOPS (pH = 7.2) (Van Goor, 1997). The coefficients for equation 1 were determined to be: K_d =

265.00 nM, $Q(F_{min}/F_{max}) = 1.95$, $R_{min} = 0.78$, and $R_{max} = 3.72$ (Christopher, 1997).

Immediate Effects of Treatments on Intracellular Calcium

To determine the immediate effects of various treatments, fura-2 fluorescence was monitored as solutions were perfused onto the cells using a gravity-fed, vacuum-emptied perfusion system. Images were captured at 30 second intervals. Alternatively, 1 mL aliquots of 3x solutions at 37°C were pipetted into the 2 mL of MEBS covering the cells, and fura-2 fluorescence was then monitored at 20 second intervals. The ability of the fura-2/AM loaded P388D.1 cells to give typical calcium responses was verified by treating macrophage cultures with 333 nM ionomycin and perfusing separate cultures with 20 ng/mL platelet activating factor.

Activation of Macrophages

For the 24 hour incubation, macrophages were suspended in complete DMEM at a density of 1.0×10^6 cells/mL, transferred to 12 x 75 mm polypropylene tubes (Becton Dickinson Labware, Lincoln Park, NJ), and treated with DMEM, 25 U/mL IFN-γ, 1 µg/mL LPS, or 25 U/mL IFN-γ and 1 µg/mL LPS. The cells were incubated at 37°C and 5% CO_2 for 24 hours, transferred to CI dishes, and then loaded with fura-2/AM. For the one hour treatment, macrophages were suspended in complete DMEM at a density of 1.0×10^5 cells/mL, 400 µL aliquots were seeded onto CI dishes (4.0×10^4 cells), allowed to attach at 37°C and 5% CO_2 for 1 hour, and treated with fura-2/AM loading medium containing DMEM, 100 U/mL IFN-γ, 1 µg/mL LPS, or 100 U/mL IFN-γ and 1 µg/mL LPS for 1 hour at 37°C and 5% CO_2. The cells were washed, and viewed as above. The $[Ca^{2+}]_i$ of approximately 90 cells from 3 separate calcium imaging dishes were determined for every treatment group (DMEM, IFN-γ, LPS, and LPS/IFN-γ) in every trial. The $[Ca^{2+}]_i$ was determined for a rectangle within the cytoplasm of each cell that was clearly outside of the nucleus and that was typical of the $[Ca^{2+}]_i$ for the rest of the cytoplasm.

Statistical Analyses

The distributions of the intracellular calcium concentrations of resting and activated B10-4(S) and P388D.1 cells were compared using a Kolmogorov-Smirnov test from Statview 4.01 software for the Power Macintosh. The rest of the data were analyzed with one factor analyses of variance followed by a least square means test between treatment groups using Super-ANOVA software for the Power Macintosh. A BonFerroni correction was applied to all analyses using multiple comparisons, and the p value that was considered to be significant was equal to α/n, where α was the overall desired level of significance for the experiment and n was the number of comparisons. An α less than 0.05 was considered to be statistically significant.

Results

Loading Macrophages with Fura-2/AM and Validation of $[Ca^{2+}]_i$ Measurements

The P388D.1 cell line loaded well with fura-2/AM, giving a diffuse pattern of fluorescence in the cytoplasm of cells and little fluorescence in the nucleus (Figs. 2-1b and 2-1c). The B10-4(S) cell line loaded almost as well except there was a greater tendency for compartmentalization that resulted in a larger number of cells with bright specks in the cytoplasm (Figs. 2-2b and 2-2c). Treatment of P388D.1 cells with the calcium ionophore ionomycin resulted in the expected response of a rapid increase in intracellular calcium in the cells followed by a slow return to average values (Figs. 2-3 and 2-4). In both cell lines, the $[Ca^{2+}]_i$ of untreated cells was relatively stable, with only minor oscillations upward or downward from a specific value. Treating the P388D.1 macrophages with the chemotactic peptide platelet activating factor (PAF) caused a rapid transient increase in $[Ca^{2+}]_i$ similar to those seen by other researchers (Figs. 2-5 and 2-6) (Randriamampita and Trautmann, 1989, Katnik and Nelson, 1993). The B10-4 cell line gave similar responses (data not shown). It should be noted that the reported $[Ca^{2+}]_i$ values of the macrophages may have consistently varied from the actual $[Ca^{2+}]_i$ of the cells because the calibration of the calcium imaging system was carried out at 20 °C whereas experiments were conducted

at 37 °C. Nevertheless, relative changes in $[Ca^{2+}]_i$ would not have been affected. As such, the P388D.1 and B10-4 cells loaded well enough with fura-2/AM to measure the $[Ca^{2+}]_i$ of resting macrophages and to determine changes induced by treatments with calcium mobilizing agents. The system was then optimized so that the $[Ca^{2+}]_i$ of the macrophages could be examined.

Effect of a 24 Hour Activation Treatment on the $[Ca^{2+}]_i$ of Macrophages

The $[Ca^{2+}]_i$ of resting P388D.1 cells was 221.45 ± 5.72 nM (mean ± SEM) (Fig. 2-7A). Treatment with 25 U/mL IFN-γ and 1 μg/mL LPS for 24 hours caused a significant ($p<0.0001$) shift in the population distribution of the P388D.1 cells' $[Ca^{2+}]_i$ to a mean of 130.59 ± 3.77 nM (Fig. 2-7B). In comparison, the $[Ca^{2+}]_i$ of resting B10-4(S) cells had a significantly lower mean of 164.59 ± 4.43 nM (Fig. 2-8A). As with the P388D.1 cell line, treatment with 25 U/mL IFN-γ and 1 μg/mL LPS for 24 hours caused a significant ($p<0.0001$) shift in the population distribution of the B10-4(S) $[Ca^{2+}]_i$ to a mean of 133.29 ± 4.51 nM (Fig. 2-8B).

When the P388D.1 cells were treated for 24 hours with IFN-γ or LPS alone, 25 U/mL IFN-γ had no effect on the $[Ca^{2+}]_i$ while 1 μg/mL LPS caused a significant reduction ($p<0.001$) in the mean $[Ca^{2+}]_i$ when compared to the DMEM control (Fig. 2-9). In the experiments on P388D.1 cells, the data from the two trials examining the effects of individual activator treatment on the $[Ca^{2+}]_i$ were similar and the data sets were combined when analyzed. The same was not true of the B10-4(S) cell line. In the first trial, both the 1 μg/mL LPS and the 25 U/mL IFN-γ/1 μg/mL LPS treatments had significantly ($p<0.05$) higher mean $[Ca^{2+}]_i$ than the DMEM control (Fig. 2-10A). In the second trial, the 25 U/mL IFN-γ treatment had a significantly ($p<0.002$) higher mean $[Ca^{2+}]_i$, while the 1 μg/mL LPS treatment had a significantly ($p<0.05$) lower mean $[Ca^{2+}]_i$ than the DMEM control cells. These contradictory results likely reflect the reduced loading efficacy of the B10-4(S) cells for fura-2/AM than the P388D.1 cells combined with greatly increased noise through the

CCD camera during the last portion of experimentation. Since the P388D.1 cells loaded better with fura-2/AM to give a brighter signal, the increased noise made little difference in determining the intracellular calcium concentration of the P388D.1 cells, but the increased noise made determination of accurate $[Ca^{2+}]_i$ for the B10-4(S) cells impossible. Therefore, no further experiments were performed on the B10-4(S) cell line.

Effect of a One Hour Activation Treatment on the $[Ca^{2+}]_i$ of P388D.1 Cells

The $[Ca^{2+}]_i$ of resting macrophages at one hour post-treatment was very similar to that observed at 24 hours with a mean of 237.22 ± 7.57 nM (Fig. 2-11). A one hour treatment with 100 U/mL IFN-γ did not significantly affect the $[Ca^{2+}]_i$ of the P388D.1 cells, whereas treatment with either 1 μg/mL LPS or 100 U/mL IFN-γ and 1 μg/mL LPS significantly ($p<0.05$) lowered the mean $[Ca^{2+}]_i$ of the P388D.1 cells to 219.511 ± 4.34 nM and 217.39 ± 4.93 nM, respectively (Fig. 2-11).

Discussion

The cytotoxic activator LPS, with or without IFN-γ, was clearly capable of affecting the intracellular calcium concentration of both the B10-4(S) and P388D.1 cell lines after a long-term 24 hour incubation. However, since the decrease in $[Ca^{2+}]_i$ was observed so long after the cytotoxic metabolic machinery was initiated at 6-8 hours post-treatment, it appeared that the altered basal calcium level caused by the cytotoxic activators was a by-product of activation rather than being essential to the activation process (Stuehr and Marletta, 1987; Neumann and Belosevic, 1996). Nevertheless, the results of experiments examining the $[Ca^{2+}]_i$ of P388D.1 cells one hour after activation showed that the reduction in $[Ca^{2+}]_i$ occurred early enough to affect induction of the cytotoxic metabolic machinery. Although the decrease in the mean $[Ca^{2+}]_i$ of the activated P388D.1 cells was smaller one hour post-treatment than at 24 hours post-treatment, the reduction was still significant ($p<0.05$) for the LPS and LPS/IFN-γ groups. Unfortunately, the many calcium-dependent

signaling processes, such as ion channel gating, protein kinase C activation, and calmodulin activation, all require increases in the cytoplasmic calcium concentration for the induction of their activity, not the observed decrease (Reiner, 1994; Xie and Nathan, 1994).

An interesting possibility is that the reduced $[Ca^{2+}]_i$ results from the cell vigorously buffering and storing calcium to levels below the normal resting level following a transient increase in $[Ca^{2+}]_i$ during macrophage activation (Scharff and Foder, 1993). Treatment with ionomycin and PAF both induced a transient rapid increase in the $[Ca^{2+}]_i$, but this increase was shortly followed by a reduction to lower $[Ca^{2+}]_i$ values as the cell's buffering capabilities were recruited. Therefore, the macrophages may undergo an increase in $[Ca^{2+}]_i$ following activation with LPS and IFN-γ that affects the intracellular signaling processes of the cell that is immediately followed by a decrease in $[Ca^{2+}]_i$ (Letari *et al.*, 1991b; Raddassi *et al.*, 1994; Rotnes *et al.*, 1994). This might explain why pharmacological agents that increase $[Ca^{2+}]_i$, such as calcium ionophore A23187 and thapsigargin, are capable of priming macrophages for subsequent triggering during the activation process while explaining the observed decrease in $[Ca^{2+}]_i$ (Raddassi *et al.*, 1994; Jun *et al.*, 1996; Park *et al.*, 1996).

A surprising finding was that the triggering agent LPS was actually responsible for alteration of the $[Ca^{2+}]_i$ rather than IFN-γ, which is a priming agent of macrophage activation. However, 1 μg/mL LPS alone was capable of fully activating macrophages for cytotoxic functions, such as production of nitric oxide, whereas 25 U/mL IFN-γ was not (Chapter 3). Therefore, the altered $[Ca^{2+}]_i$ may be related to processes in the overall activation pathway for macrophages rather than being specific for either the priming or triggering stage.

The proposed buffering-induced reductions in $[Ca^{2+}]_i$ is clearly causing a long-term alteration in the metabolism of calcium since it can be observed not only one hour after

activator treatment but also at 24 hours post-treatment when the upregulation of cytotoxic processes has already occurred. The extended length of this change would suggest that rather than purely being a byproduct of an increased $[Ca^{2+}]_i$, the decrease in $[Ca^{2+}]_i$ may play a physiological role in the activation process. For example, the decrease in $[Ca^{2+}]_i$ could shut off pathways and metabolic functions not required for cytotoxic activity after the speculative rapid and transient increase in calcium concentration has activated the cytotoxic machinery. In addition, the decrease in $[Ca^{2+}]_i$ could upregulate processes required for cytotoxic activation by removing the activity of repressing enzymes that maintain proteins in a non-functional state. An actual physiological function of the reduced basal intracellular calcium concentration and reduced activity of non-cytotoxic machinery at reduced calcium levels remains to be elucidated.

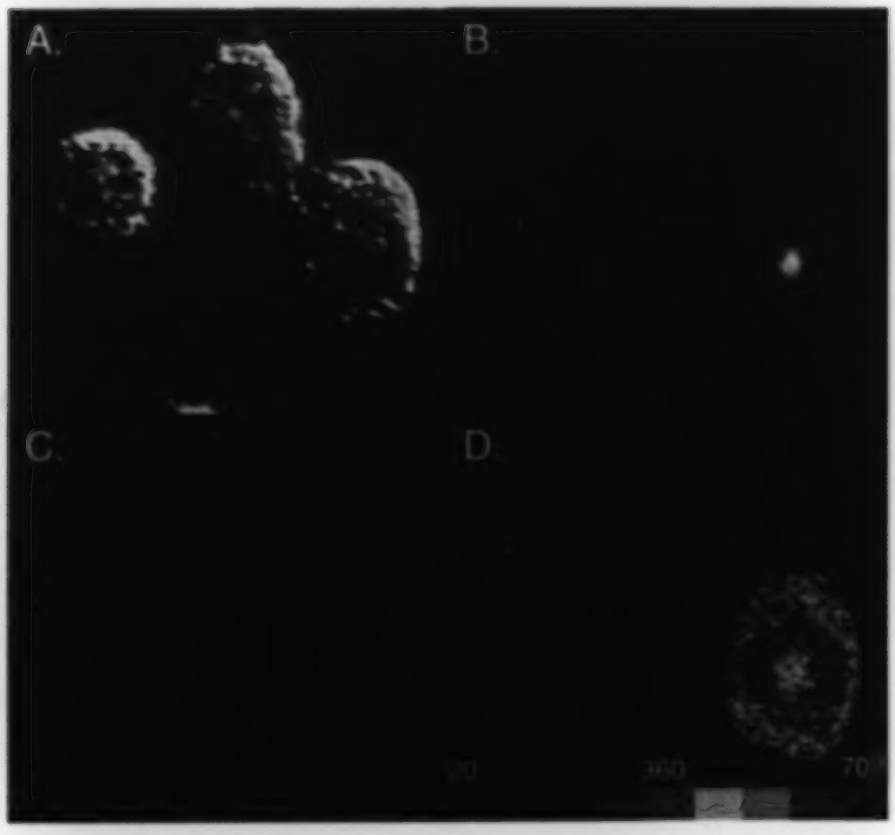

Figure 2-1. Loading of P388D.1 Cells with Fura-2/AM. P388D.1 cells as seen under differential interference contrast (A.), 340 nm excitation (B.), 380 nm excitation (C.), and false colour image showing intracellular calcium (D.). The colour bar indicates $[Ca^{2+}]_i$ ranging from 20 nM to 700 nM.

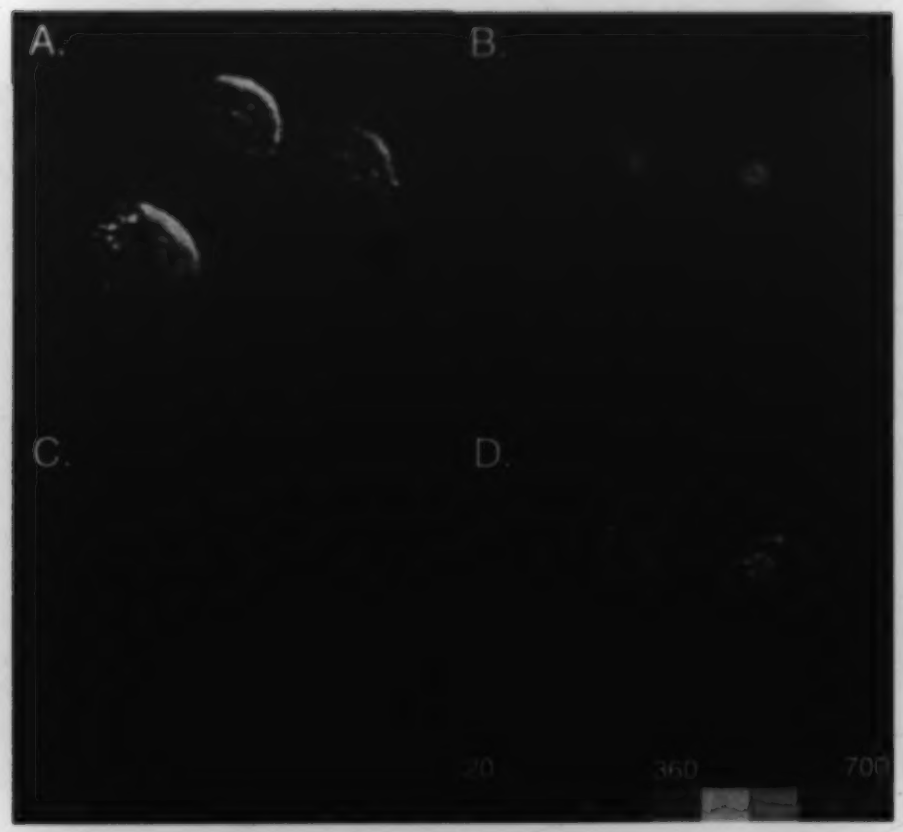

Figure 2-2. Loading of B10-4(S) Cells with Fura-2/AM. B10-4 cells as seen under differential interference contrast (A.), 340 nm excitation (B.), 380 nm excitation (C.), and false colour image showing intracellular calcium (D.). The colour bar indicates $[Ca^{2+}]_i$ ranging from 20 nM to 700 nM.

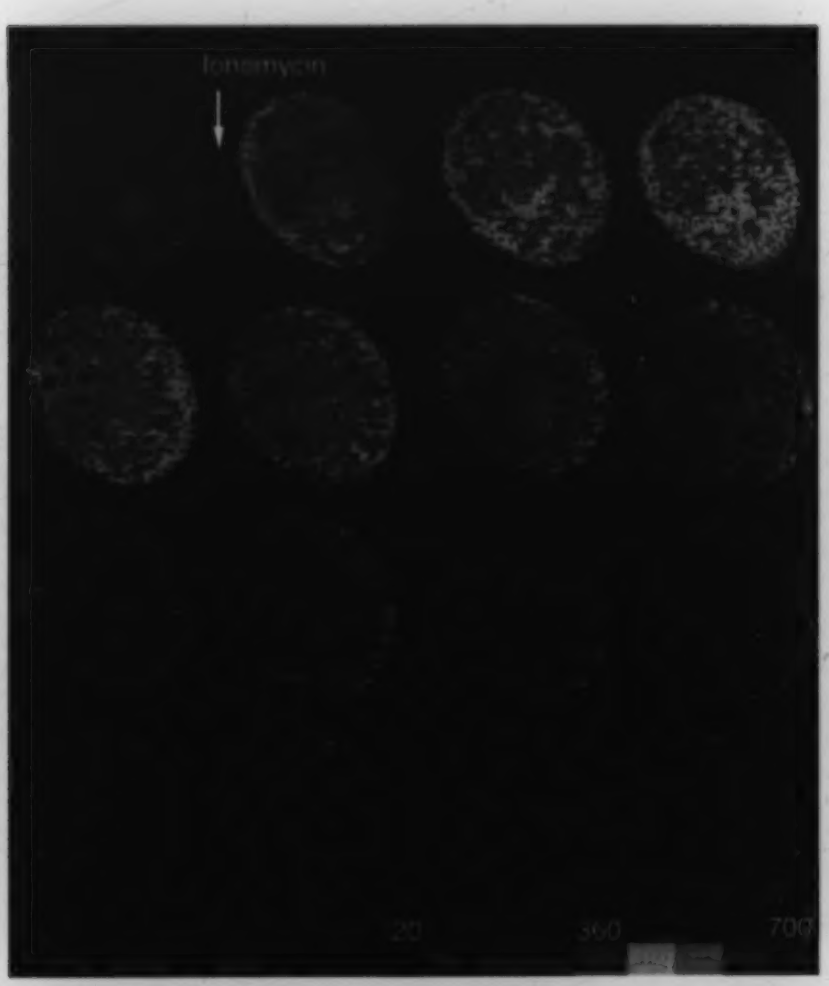

Figure 2-3. Ionomycin increases $[Ca^{2+}]_i$. Treating P388D.1 cells with 333 nM ionomycin induces a rapid increase in intracellular calcium that eventually returns to basal levels. Calcium monitored at 20 second intervals shown sequentially from left to right. The colour bar indicates $[Ca^{2+}]_i$ ranging from 20 nM to 700 nM.

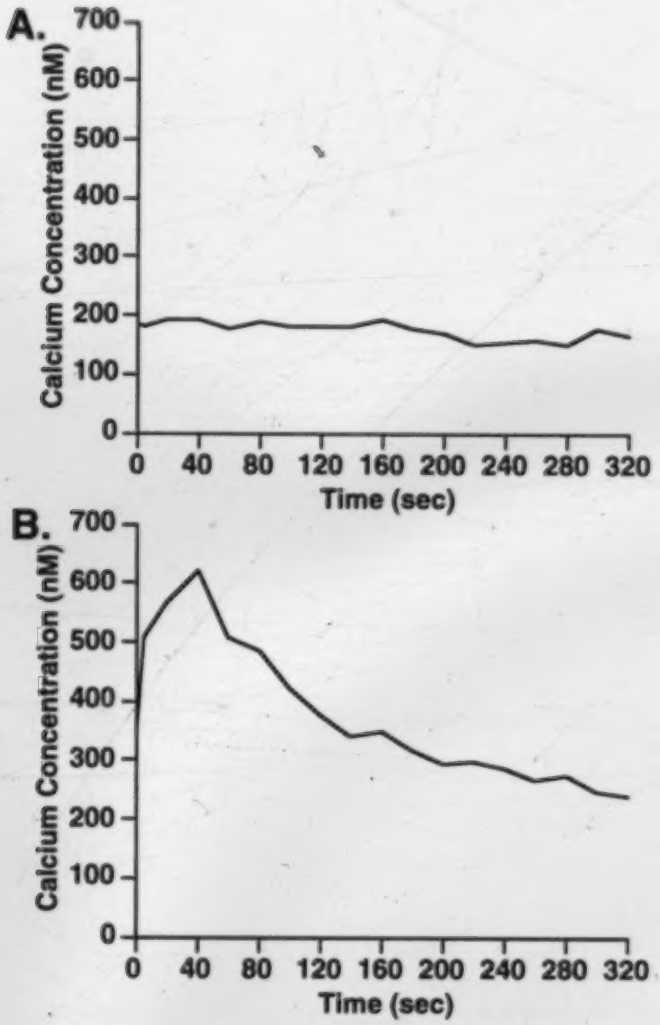

Figure 2-4. Time course of the intracellular calcium concentration of a resting P388D.1 cell (A.) and a 333 nM ionomycin-treated P388D.1 cell (B.). Measurements were taken at 20 second intervals, and ionomycin was applied at time 0.

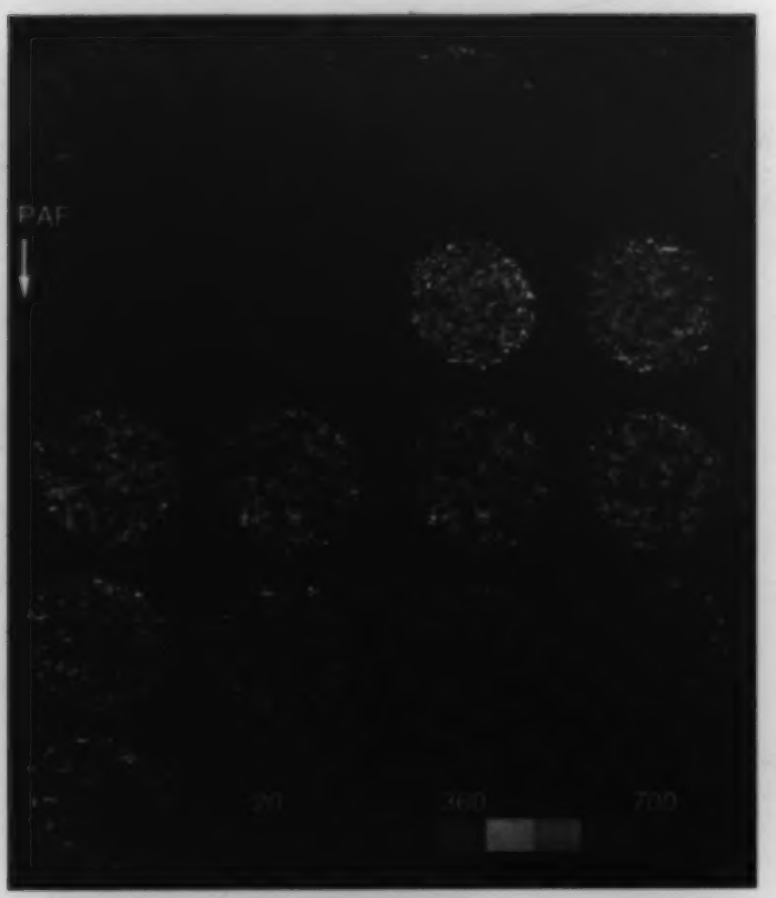

Figure 2-5. PAF increases $[Ca^{2+}]_i$. Perfusing P388D.1 cells with 20 ng/mL PAF induced a rapid increase in intracellular calcium 60 seconds after treatment that returned to basal levels. $[Ca^{2+}]_i$ monitored at 30 second intervals shown sequentially from left to right. The colour bar indicates $[Ca^{2+}]_i$ ranging from 20 nM to 700 nM.

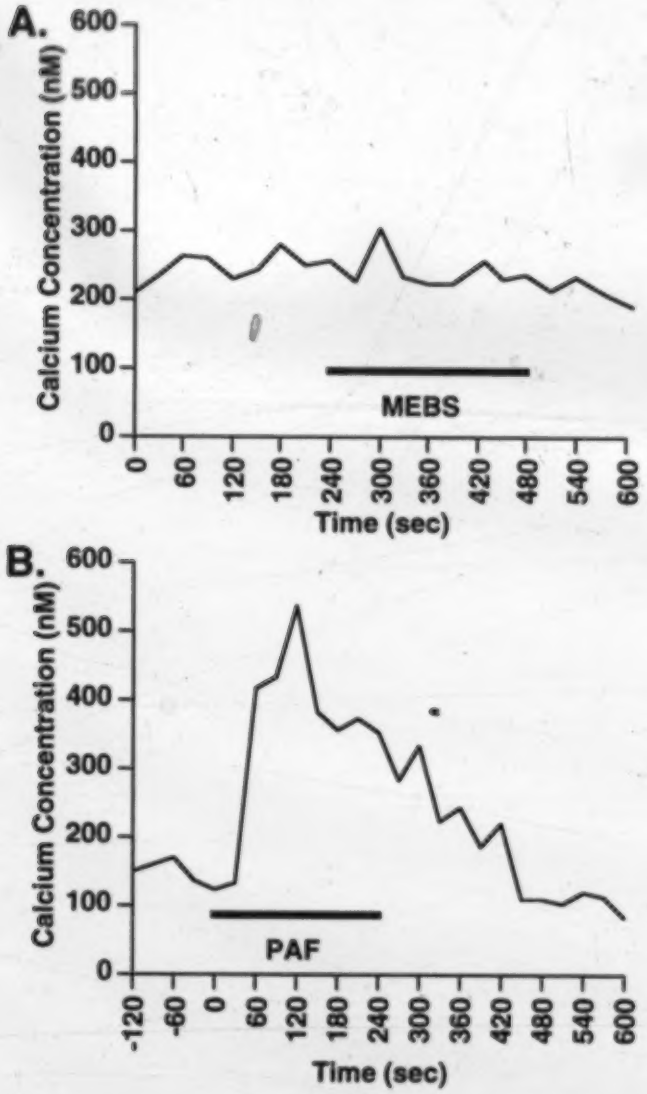

Figure 2-6. Effects of perfusion with MEBS (control) (A) or 20 ng/mL PAF (B) on the intracellular calcium concentration of individual P388D.1 cells loaded with fura-2/AM. The $[Ca^{2+}]_i$ was monitored at 30 second intervals, and the bars represent the duration of MEBS or PAF application.

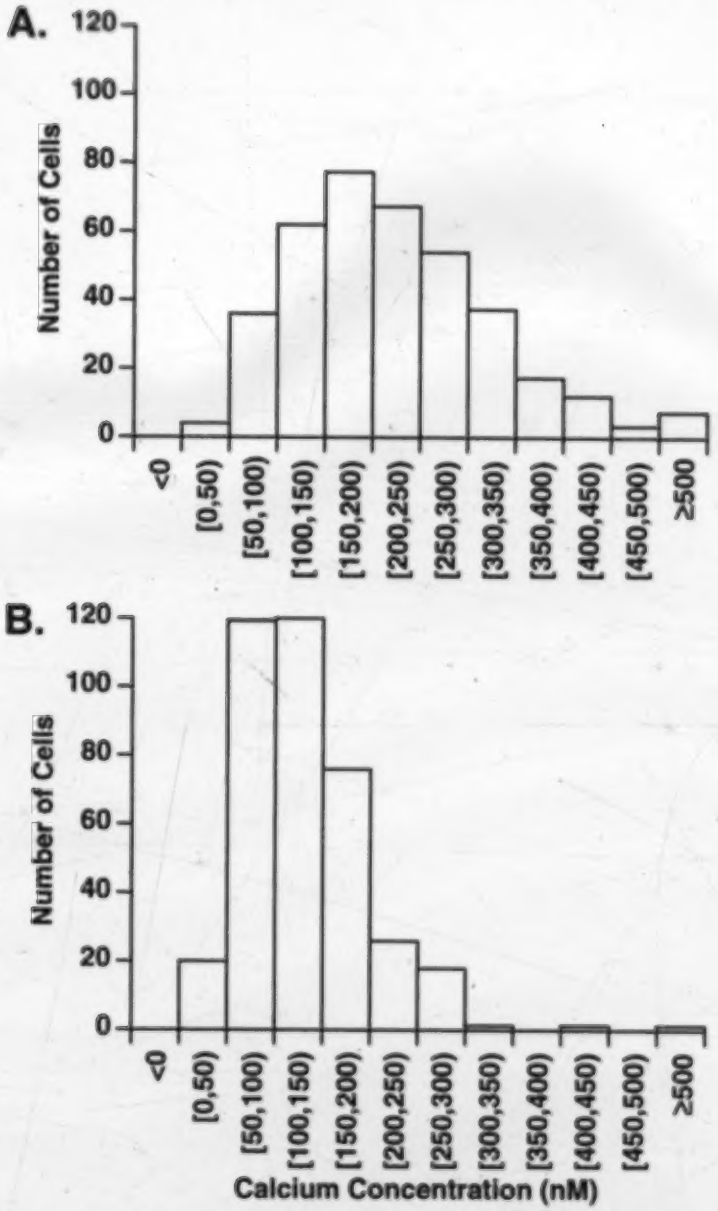

Figure 2-7. Distribution of the $[Ca^{2+}]_i$ of P388D.1 cells treated for 24 hours with DMEM (control) (A.) or 25 U/mL IFN-γ and 1 μg/mL LPS (B.). Treatment with LPS and IFN-γ caused a significant shift in the distribution of calcium concentrations to lower values ($p<0.0001$, Kolmogorov-Smirnov test, N=377 DMEM, N=382 LPS/IFN-γ).

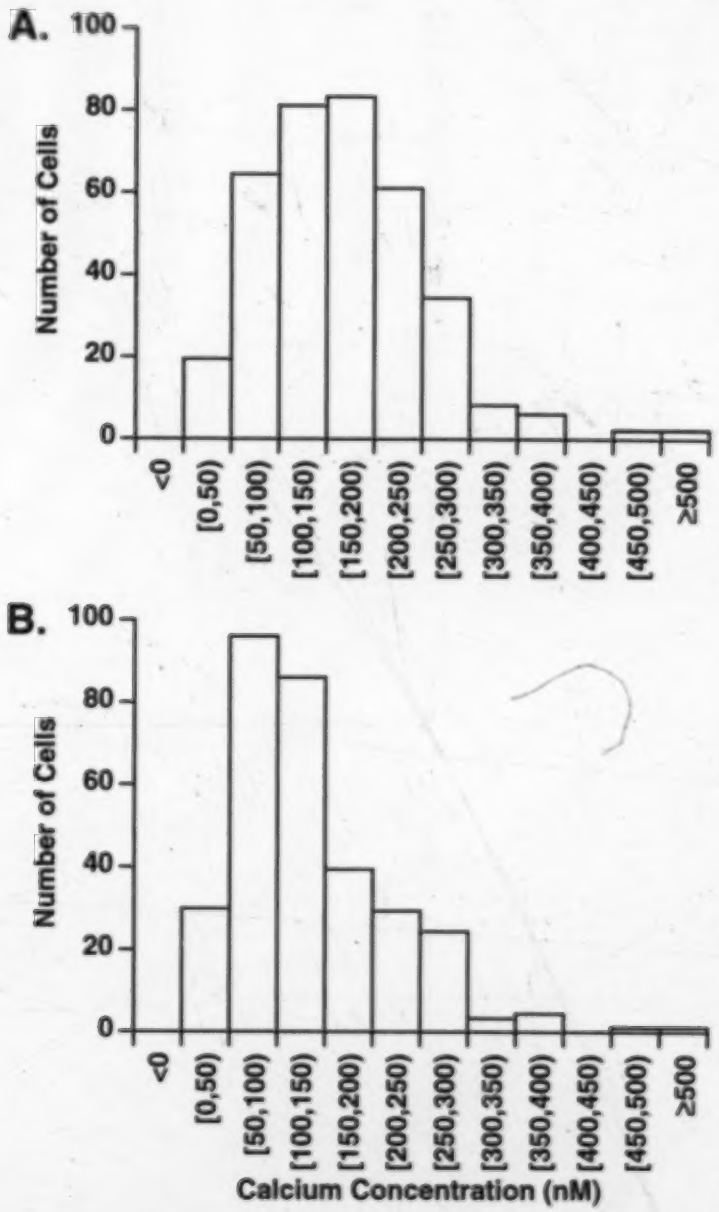

Figure 2-8. Distribution of the $[Ca^{2+}]_i$ of B10-4(S) cells treated for 24 hours with DMEM (control) (A.) or 25 U/mL IFN-γ and 1 μg/mL LPS (B.). Treatment with LPS and IFN-γ caused a significant shift in the distribution of calcium concentrations to lower values ($p<0.0001$, Kolmogorov-Smirnov test, N=360 DMEM, N=313 LPS/ IFN-γ).

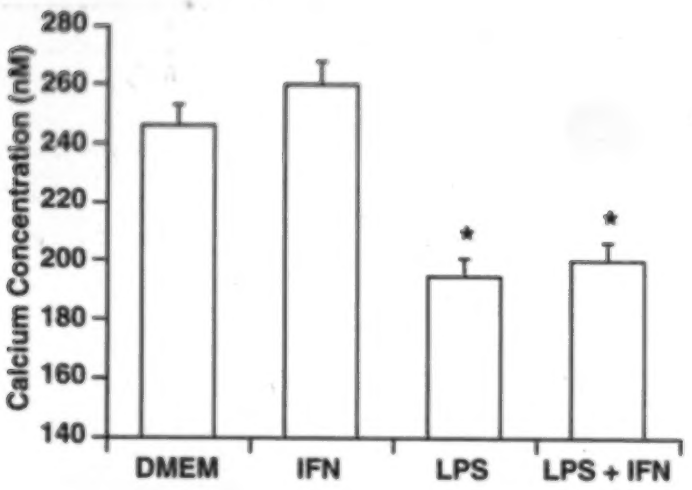

Figure 2-9. Long-term effects (24 hour) of treating with DMEM (control), 25 U/mL IFN-γ, 1 μg/mL LPS, or 25 U/mL IFN-γ and 1 μg/mL LPS on the $[Ca^{2+}]_i$ in P388D.1 (combined data from 2 trials). An * indicates a significant difference from the DMEM control ($p<0.001$, least square means of a one-factor ANOVA).

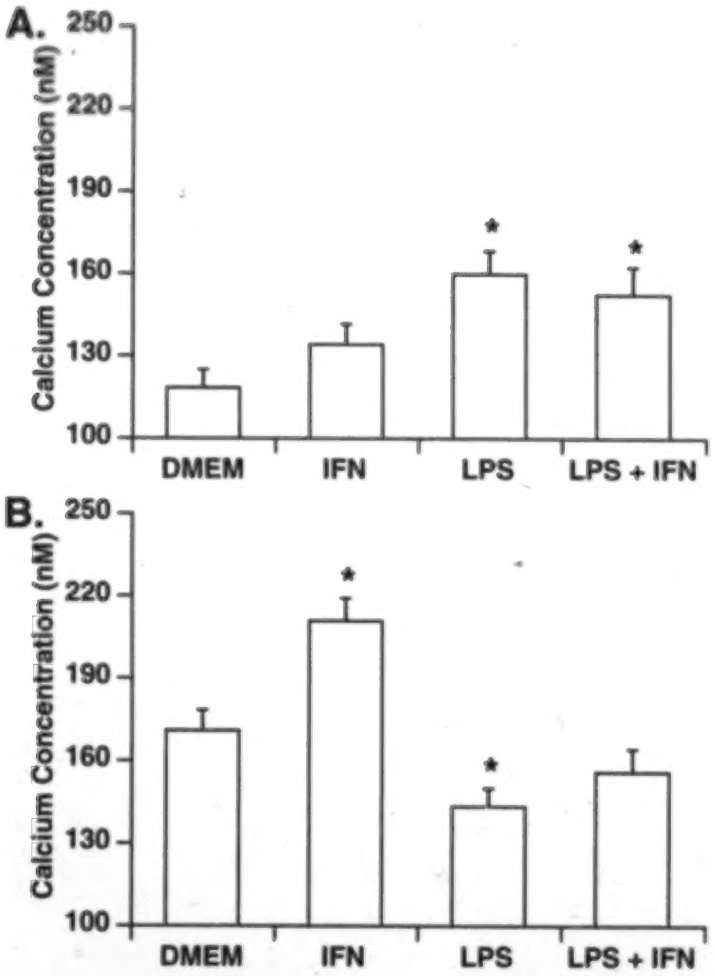

Figure 2-10. Long-term effects (24 hour) of treating with DMEM (control), 25 U/mL IFN-γ, 1 μg/mL LPS, or 25 U/mL IFN-γ and 1 μg/mL LPS on the $[Ca^{2+}]_i$ in B10-4(S) cells from experimental trials 1 (A.) and 2 (B.). An * indicates a significant difference from the DMEM control ($p<0.05$, least square means of a one-factor ANOVA).

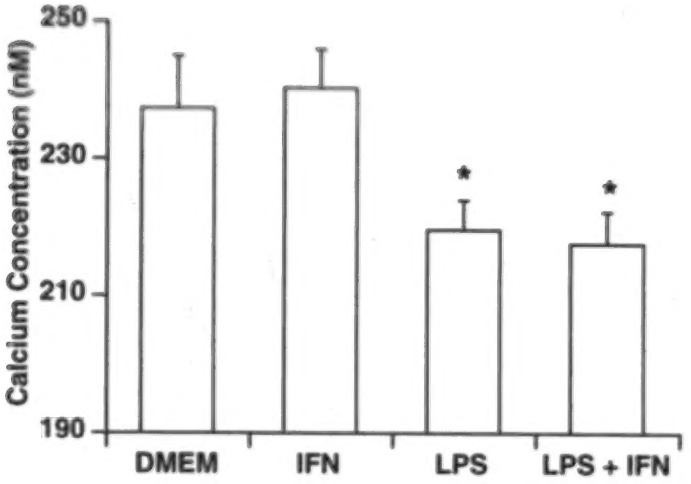

Figure 2-11. Short-term effects (one hour) of treating with DMEM (control), 25 U/mL IFN-γ, 1 μg/mL LPS, or 25 U/mL IFN-γ and 1 μg/mL LPS on the $[Ca^{2+}]_i$ in P388D.1 cells (combined data from 2 of 3 trials). An * indicates a significant difference from the DMEM control ($p<0.05$, least square means of a one-factor ANOVA).

Chapter 3 : Involvement of Potassium Channels in the Nitric Oxide Production of Macrophages

Introduction

Production of nitric oxide (NO) is an important antimicrobial response macrophages use to eliminate microbial and tumoral targets and is essential for the resolution of infection by a number of obligate intracellular parasites and microbes (Nathan and Hibbs, 1991; Green *et al.*, 1990; Flesch and Kaufmann, 1991; Reiner; 1994; de Groote and Fang, 1995). The cytotoxicity of nitric oxide, and other reactive nitrogen molecules, results from the molecule's high affinity for iron that allows it to combine with and disrupt key moieties in enzymes involved in the respiratory cycle, intracellular signaling, and the synthesis of DNA (Moncada and Higgs, 1993; Neumann and Belosevic, 1996). Among the affected enzymes are the oxidoreductases of the mitochondrial electron transport chains, aconitase, protein kinase C, ferritin, indoleamine 2,3-dioxygenase, and ribonucleotide reductase (Neumann and Belosevic, 1996). Within activated macrophages, nitric oxide is produced by the inducible isoform of the nitric oxide synthase enzyme (iNOS) that catalyses the oxidation of the terminal guanidino nitrogens donated by L-arginine (Green *et al.*, 1990; Xie and Nathan, 1994; Neumann and Belosevic, 1996). The cloned iNOS enzyme exists as a dimer of 130-150 kDa monomers that requires flavin mononucleotide (FMN), flavin-adenine dinucleotide (FAD), nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopterin, and heme to function at full efficiency (Xie *et al.*, 1992; Marletta, 1993; Baek *et al.*, 1993). Regulation of iNOS activity was initially thought to be under controls similar to those regulating the constitutive isoform of nitric oxide synthase because both isoforms bound calmodulin (Xie *et al.*, 1992). Changes in cytoplasmic calcium concentration alter the ability of calmodulin to bind and activate cNOS, but the iNOS enzyme has a very high binding affinity for calmodulin that allows it to bind calmodulin at

the very low Ca^{2+} concentrations found within resting macrophages (Xie and Nathan, 1994). Since calmodulin is already bound to and activating iNOS, transients of Ca^{2+} within the cytosol of macrophages do not alter enzyme activity. Rather, regulation of iNOS activity appears to occur at the level of transcription by selective induction of the iNOS gene and degradation of iNOS mRNA transcripts because the amount of nitric oxide produced is directly proportional to the amount of iNOS mRNA in the cytoplasm (Weisz *et al.*, 1994).

The expression of iNOS, which induces nitric oxide production, is a carefully regulated two-stage process. Like many of the cytotoxic mechanisms of the macrophage, nitric oxide is indiscriminate in its effects on cells, damaging both host tissues and target cells (Lorsbach *et al.*, 1993; Neumann and Belosevic, 1996; Park *et al.*, 1996). To minimise damage to self tissues, a priming stage and a triggering stage must occur before the cell is capable of full nitric oxide production (Adams and Hamilton, 1984; Crawford *et al.*, 1994). The priming stage occurs when soluble mediators, such as the cytokine interferon-γ (IFN-γ), binds to receptors embedded in the plasma membrane of the macrophage and induces intracellular signaling cascades (Raddassi *et al.*, 1994; Jun *et al.*, 1996; Park *et al.*, 1996). These signaling cascades ultimately alter the expression of enzymes, including inducible nitric oxide synthase, and receptor proteins to prepare the metabolic machinery to initiate a cytotoxic response. The binding of another soluble mediator, often a molecule of the invading microbe such as bacterial lipopolysaccharide (LPS), to receptors on the macrophage starts the triggering stage by initiating another series of signaling cascades that activates the previously "primed" cytotoxic machinery (Jun *et al.*, 1994; Jun *et al.*, 1996; Park *et al.*, 1996).

The signaling cascades involved in the activation of nitric oxide production by IFN-γ and LPS have been only partially characterised. Although the receptors for soluble mediators have not been identified, it is believed that priming agents induce an increase in

the cytoplasmic calcium concentration of the macrophages, presumably by the production of inositol 1,4,5-triphosphate (IP_3) by receptor-activated phospholipase C. This increase in $[Ca^{2+}]_i$ is primarily due to the release of Ca^{2+} from intracellular stores (Jun *et al.*, 1996). The importance of this increase is suggested by the ability of agents that increase intracellular calcium, such as the calcium ionophore A23187 and the endoplasmic reticulum Ca^{2+}-ATPase inhibitor thapsigargin, to prime macrophages for subsequent triggering of NO production with LPS (Raddassi *et al.*, 1994; Jun *et al.*, 1996; Park *et al.*, 1996). The triggering stage is believed to involve the activity of protein kinase C (PKC) because treatment with PKC-activating phorbol esters is sufficient to trigger NO production in macrophages previously primed with IFN-γ or thapsigargin (Hortelano *et al.*, 1993; Jun *et al.*, 1994; Jun *et al.*, 1996). Alterations in cytosolic calcium concentrations and activation of PKC are only two of the many intracellular signaling cascades involved in macrophage activation. Whereas, the other intervening signaling cascades have yet to be characterised, their ultimate collective activity is the covalent modification of nuclear factors responsible for altering gene expression in the macrophage nucleus (Aderem, 1993; Introna *et al.*, 1986; Eilers *et al.*, 1993).

The regulatory regions of the iNOS gene and nuclear factors capable of affecting its expression have been identified. Transcription of the iNOS gene is enhanced by the binding of nuclear factors to two discrete upstream regulatory regions (Lowenstein *et al.*, 1993). Treatment with IFN-γ and LPS induces a higher rate of iNOS mRNA transcription and increases the stability of the iNOS mRNA transcripts (Lorsbach *et al.*, 1993; Weisz *et al.*, 1994). The increased transcription rate is due to different transcription factors binding to two distinct regulatory regions of the iNOS gene. The first regulatory region binds to transcription factors induced by the triggering agent LPS, such as nuclear factor interleukin 6 (NF-IL6) and NF-κB, and can increase expression of iNOS by up to 75-fold

(Lowenstein *et al.*, 1993). The second region binds to IFN-γ-related transcription factors, and in conjunction with region I, increases transcription by an additional 10-fold (Lowenstein *et al.*, 1993). This provides a direct method for priming and triggering agents to synergise in the induction of macrophage cytotoxic functions, and suggests a method of how priming and triggering can affect the expression of cytotoxic function in macrophages.

Experiments examining the effects of potassium channel inhibitors on the cytotoxic functions of a variety of leukocytes has suggested that potassium channels are involved in the signaling cascades responsible for inducing cytotoxic functions (Chapter 1 - Schlicter *et al.*, 1986; Gallin, 1991; Damjanovich *et al.*, 1992). Ion channels are integral membrane proteins or protein complexes that control the passage of specific ions into or out of the cell (Hille, 1992). Ion channels specific for calcium have direct effects on the intracellular signaling pathways and gene expression of cells by altering the cytoplasmic calcium concentration (AlMohanna and Hallet, 1988; Scharff and Foder, 1993). However, the major role of most ion channels within cells, particularly those specific for potassium ions, is the establishment and maintenance of the membrane potential (Gallin, 1991). There is growing evidence that alterations in the activity of ion channels and the resulting changes in membrane potential can have significant effects on other proteins and enzymes embedded in the plasma membrane (Artalejo *et al.*, 1992; Haslberger *et al.*, 1992). This includes a number of intracellular signaling protein complexes responsible for propagating signals that activate macrophages and provides a mechanism that the inhibitors of ion channels may use to affect the cytotoxic function of macrophages. In this chapter, I report on experiments that examined whether alterations in the activity of potassium channels caused by specific pharmacological blockers and activators affected the nitric oxide production and cell viability of the P388D.1 and B10-4(S) macrophage-like cell lines.

Materials and Methods

Reagents

Recombinant murine interferon-γ (IFN-γ) was purchased from Genzyme (Boston, MA) and lipopolysaccharide (LPS) from *Salmonella typhimurium* was obtained from Difco Laboratories (Detroit, MI). Quinine hydrochloride, cobalt chloride, 4-aminopyridine, tetraethylammonium chloride, and minoxidol were obtained from Sigma Chemical Company (St. Louis, MO).

Cell Lines and Media

Two cell lines with different growth and cytotoxic activation characteristics were obtained for experimentation. The macrophage-like cell line P388D.1 consisted of slower growing cells at a later stage of differentiation, and was obtained from the American Type Culture Collection (Koren *et al.*, 1975). The B10-4(S) macrophage-like cell line consisted of rapidly dividing cells with morphology typical of less mature macrophages, and was a kind gift from Dr. D. Radzioch, McGill University. The B10-4(S) cell line was immortalized from bone-marrow derived macrophages isolated from C57BL/10.A (B10A.Bcg^S) mice that were susceptible to Bacillus Calmette Guerin (Radzioch *et al.*, 1991). Both cell lines were cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium containing 50 μg/mL gentamicin (Gibco BRL, Grand Island, NY) and 10% fetal bovine serum (HyClone Laboratories Inc., Logan, UT) (complete DMEM).

Activator Dose Response for LPS and IFN-γ

The optimal concentrations of the cytotoxic activators LPS and IFN-γ were determined by assessing the nitric oxide response at different concentrations of activators. Macrophages seeded into 96-well culture plates at a density of 1.0×10^5 cells/well were treated with 100 ng/mL to 10 μg/mL LPS, with or without 25 U/mL IFN-γ, or with 5 U/mL to 200 U/mL IFN-γ, with or without 500 ng/mL LPS. Each treatment concentration was done in triplicate, and a nitric oxide assay and a viability assay were done on parallel

cultures of macrophages for every treatment group. The treated macrophage cultures were incubated at 37 °C and 5% CO_2 for 24 hours prior to performing the appropriate assays. These experiments were repeated three times.

Activation of Macrophages and Treatment with Modulators of Potassium Channels

Macrophages of the B10-4(S) and P388D.1 cell lines were seeded into the wells of 96-well tissue culture plates at a density of 1.0×10^5 cells/well and allowed to attach to the bottom of the wells at 37 °C and 5% CO_2 for 1 hour. Macrophages in different culture plates were treated with cobalt chloride (25 µM to 500 µM), tetraethylammonium (500 µM to 10 mM), 4-aminopyridine (50 µM to 2 mM), quinine (5 µM to 100 µM), and minoxidol (100 nM to 5 µM) in conjunction with complete DMEM (control) or 1 µg/mL LPS and 25 U/mL IFN-γ. Each treatment concentration was done in triplicate, and a nitric oxide assay and a viability assay were done on parallel cultures of macrophages for every treatment group. The treated macrophage cultures were incubated at 37 °C and 5% CO_2 for 24 hours prior to performing the appropriate assays. These experiments were repeated three times.

Time Course of the Nitric Oxide Production and Respiratory Viability

Macrophages were seeded at a density of 5.0×10^4 cells/well, and wells of triplicate macrophage cultures were treated with 10 mM TEA, 1 mM 4-AP, or 50 µM quinine in conjunction with complete DMEM (control) or 1 µg/mL LPS and 25 U/mL IFN-γ. A nitric oxide assay and a viability assay were done on parallel cultures of macrophages for each time point examined: 0, 6, 12, 24, 48 and 72 hours. These experiments were repeated twice.

Pre-Treatment of Macrophages with Cytotoxic Activators or Potassium Channel Inhibitors

Macrophages were seeded at a density of 1.0×10^5 cells/well, and wells of triplicate macrophage cultures were pre-treated for 6 hours at 37 °C and 5% CO_2 with DMEM (control), 1 µg/mL LPS, 25 U/mL IFN-γ, LPS and IFN-γ, or with the inhibitors 10 mM

TEA, 1 mM 4-AP, and 50 µM quinine. The macrophage cultures were then fully activated by addition of 1 µg/mL LPS and/or 25 U/mL IFN-γ, as required, and potassium channel activity was inhibited by addition of 10 mM TEA, 1 mM 4-AP, or 50 µM quinine, again as required. The cultures were incubated at 37 °C and 5% CO_2 for a further 24 hours. These experiments were repeated twice.

Nitric Oxide Assay

The nitric oxide production of the macrophages was indirectly determined by using a colorimetric assay, the Griess reaction, to measure the accumulation of the stable end product of nitric oxide degradation, nitrite, within the superfusate of wells (Green *et al.*, 1992). Briefly, 100 µL volumes of macrophage superfusates were transferred from the treatment plate wells to wells of a 96-well microtitre plate. An equal volume (100 µL) of 1% sulfanilamide (Sigma, St. Louis, MO) in 2.5% phosphoric acid (Anachemia, Montreal, Que.) was added to each well. This was followed by 100 µL of 0.1% N-naphthyl-ethylenediamine (Sigma, St. Louis, MO) in 2.5% phosphoric acid (Anachemia, Montreal, Que.). The plate was gently tapped to mix the contents, allowed to sit for 2 minutes, and the optical density at 540 nm (O.D. 540 nm) was determined using an automated spectrophotometer (Bio-tek Instruments Inc., Winooski, VT). The approximate concentration of nitrite in samples was determined from a standard curve that was generated by using known concentrations of sodium nitrite (Sigma, St. Louis, MO).

Cell Respiration (XTT Viability Assay)

Changes in macrophage viability were assessed using the XTT viability assay (Appendix 1). This assay measures the respiratory activity of macrophage mitochondria by determining the accumulation of a coloured formazan byproduct within the superfusates of treated wells (Scuderio *et al.*, 1988; Roehm *et al.*, 1991; Stevens and Olsen, 1993). Briefly, a 50 µL volume of 1 mg/mL sodium 3,3'-[1[(phenylamino)carbonyl]-3,4-

tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) (ICN Biomedicals Inc., Aurora, Ont.) and 50 µg/mL 2,3-dimethoxy-5-methyl-1,4-benzoquinone (CoQ) (Sigma, St. Louis, Mo.) in 1x Dulbecco's phosphate buffered saline (Gibco BRL, Grand Island, NY) was added to each well of the treatment plate. The plates were gently tapped to mix the contents, incubated for 1 hour at 37 °C and 5% CO_2, and the optical density at 450 nm (O.D. 450 nm) was determined using an automated spectrophotometer (Bio-tek Instruments Inc., Winooski, VT).

Statistics

The data were analyzed using Super-ANOVA software for the Power Macintosh. Differences between experimental groups were determined using one-factor analysis of variance and a least square means test. A BonFerroni correction was applied to all analyses using multiple comparisons, and the p value that was considered to be significant was equal to α/n, where α was the overall desired level of significance for the experiment and n was the number of comparisons. An α less than 0.05 was considered to be statistically significant.

Results

Calibration of the NO Assay

The O.D. 540 nm from the Griess reaction of increasing doses of sodium nitrite produced a nearly perfect linear curve (Fig. 3-1). A linear regression analysis of the data produced an R^2 value of 0.999 indicating that the produced linear formula (Fig. 3-1) could be used to estimate nitrite concentrations from O.D. 540 nm values.

Validation of the XTT Assay

Results from the XTT assay were correlated with cell count assays to determine whether the XTT assay was an acceptable method of determining the viability of macrophages that were resting, activated, or treated with potassium channel inhibitors (Appendix 1).

Increasing cell densities produced increasing O.D. 450 nm values from the XTT assay of resting and 25 U/mL IFN-γ/1 μg/mL LPS activated B10-4(S) and P388D.1 cells (Appendix 1). Significant correlations between changes in cell number and changes in the O.D. 450 nm of the XTT assay of macrophages treated with potassium channel inhibitors were also observed (Appendix 1). Therefore, the XTT assay was used to measure the viability of macrophages treated with potassium channel inhibitors.

Nitric Oxide Production by Activated Macrophage-like B10-4(S) and P388D.1 Cells

Treatment of 1.0×10^5 macrophages with LPS by itself for 24 hours was sufficient to induce nitric oxide production in both macrophage-like cell lines (Figs. 3-2 and 3-3). The nitric oxide production of the B10-4(S) cells increased in a dose-dependent manner until 1 μg/mL LPS where it leveled off, and addition of 25 U/mL IFN-γ had no further effect on the nitric oxide production (Fig. 3-2A). In contrast, the nitric oxide production of the P388D.1 cells increased until 4 μg/mL LPS, and addition of 25 U/mL IFN-γ produced further increases in the nitric oxide production (Fig. 3-3A). The B10-4(S) cells consistently exhibited a higher nitric oxide response than the P388D.1 cells when seeded at identical cell densities (1×10^5/well). Only the highest doses of LPS (4 -10 μg/mL) significantly reduced the cell respiration (XTT viability) of the B10-4(S) cells (Fig. 3-2B), whereas all doses of LPS increased the cell respiration of the P388D.1 cells (Fig. 3-3B). A concentration of 1 μg/mL LPS was chosen for further experimentation because it induced high nitric oxide production in both cell lines without affecting the cell respiration.

IFN-γ was unable to induce significant nitric oxide production in either macrophage cell line when used alone (Figs. 3-4 and 3-5). However, addition of 500 ng/mL LPS to the IFN-γ treatments produced increases in the nitric oxide production of both cell lines that were dependent on the concentration of IFN-γ (Figs. 3-4A and 3-5A). The dose-dependent increases in nitric oxide production leveled off at 25 U/mL IFN-γ for both the

B10-4(S) and the P388D.1 cell lines. Treating the macrophages with IFN-γ, alone or in combination with 500 ng/mL LPS, caused a decrease in cell respiration above 25 U/mL IFN-γ (Figs. 3-4B and 3-5B). A concentration of 25 U/mL IFN-γ was selected for further experimentation because of its ability to augment the nitric oxide production of both cell lines while affecting cell respiration as little as possible.

A time course of the nitric oxide production of 1 μg/mL LPS and 25 U/mL IFN-γ activated macrophages, initially seeded at 1.0×10^4 cells/well, showed that there was no significant nitrite accumulation in the superfusates of the B10-4(S) cultures for 48 hours after activation, and in the superfusates of the P388D.1 cultures for 12 hours after activation (Fig. 3-6). Thereafter, there were steady time-dependent increases in the nitric oxide production for both cell lines (Fig. 3-6). There was no significant nitric oxide accumulation in the superfusates of macrophage cultures that had not been activated with LPS and IFN-γ. The time course of the cell respiration of the activated B10-4(S) and P388D.1 macrophages showed a maintenance of cell respiration for the first 6 hours (P388D.1) or 12 hours (B10-4(S)) followed by a steady increase in the cell respiration until 48 hours after activation for P388D.1 or 72 hours after activation for B10-4(S) (Fig. 3-6)

Potassium Channel Inhibitors Alter the NO Production of Activated Macrophages

Cobalt chloride

Treating the macrophages with increasing doses of cobalt chloride ($CoCl_2$) for 24 hours produced a biphasic response in the nitric oxide production of the LPS/IFN-γ activated B10-4(S) and P388D.1 cell lines (Fig. 3-7). At the lower concentrations of cobalt chloride both cell lines experienced an enhancement of nitric oxide production (Fig. 3-7). The enhanced nitric oxide production of the B10-4(S) cells was small but significant ($p<0.05$) and peaked at 50 μM cobalt chloride (Fig. 3-7A). In contrast, there was a far greater enhancement (a doubling) in the nitric oxide production of the P388D.1 cells that peaked at

100 μM cobalt chloride (Fig. 3-7B). At the higher $CoCl_2$ concentrations, the nitric oxide production of both cell lines dramatically decreased until there was no detectable nitric oxide production at 500 μM $CoCl_2$. Treatment of macrophages with increasing concentrations of cobalt chloride resulted in a dose-dependent reduction in the cell respiration that leveled off at 250 μM $CoCl_2$ for the B10-4(S) cell line and at 100 μM $CoCl_2$ for the P388D.1 cell line (Fig. 3-7). Interestingly, the cell respirations of both the B10-4(S) cells and the P388D.1 cells were falling at the concentrations where there was an enhancement of nitric oxide production as well as at concentrations where there was an inhibition of nitric oxide production (Fig. 3-7).

TEA, 4-AP, and Quinine

A 24 hour treatment with the classical potassium channel inhibitors tetraethylammonium (TEA), 4-aminopyridine (4-AP), and quinine caused significant dose-dependent decreases in the nitric oxide production of both the B10-4(S) and P388D.1 macrophages (Figs. 3-8 to 3-10). This reduced nitric oxide output by the macrophages was not due to a reduced viability or cell number because respiration of the cells was either at the same level as controls not treated with potassium channel inhibitors, or slightly higher than control levels (Figs. 3-8 to 3-10). At a threshold dose of 5 mM, TEA significantly ($p<0.05$) reduced the nitric oxide production of the B10-4(S) cells, and NO production had decreased by 28.1% at 10 mM TEA (Fig. 3-8A). In contrast, the nitric oxide production of the P388D.1 cells was more sensitive to treatment with TEA, being significantly inhibited by 2.5 mM TEA and decreasing by 55.4% at 10 mM TEA (Fig. 3-8B). 4-AP caused significant ($p<0.05$) reductions of the nitric oxide production of both the B10-4(S) and P388D.1 cell lines at a concentration of 250 μM. However, the B10-4(S) cell line underwent a 66.3% reduction in the nitric oxide production when treated with 2 mM 4-AP whereas the the P388D.1 cell line experienced a 48.2% reduction at the same concentration (Fig. 3-9). Quinine caused a significant ($p<0.05$) reduction in the nitric oxide production of the B10-4(S) cell line at 10

μM quinine and NO production had decreased 55.4% at 100 μM quinine (Fig. 3-10A). The P388D.1 cell line's nitric oxide production was not significantly reduced until 25 μM quinine, but was reduced as much as the B10-4(S) cell line for the 100 μM quinine treatment decreasing to 55.3% (Fig. 3-10B).

Minoxidol

To verify that the effect of potassium channel inhibitors on nitric oxide production was due to specific actions on the potassium channels, the effects of the potassium channel agonist minoxidol were also assessed. Minoxidol caused significant ($p<0.05$) enhancements of the nitric oxide production in both the B10-4(S) and P388D.1 cell lines with no significant effect on cell respiration (Fig. 3-11).

Time Course of the NO Production of Inhibitor-treated B10-4(S) and P388D.1 Cells

Treatment of activated P388D.1 macrophages with 10 mM TEA caused approximately a 50 % decrease in the nitric oxide production, compared to control macrophages, by 6 hours after treatment (Fig. 3-12B). At 12 hours post-treatment there was a slight recovery in nitric oxide production, but thereafter there was a steady decline in nitric oxide production to 72 hours post-treatment (Fig. 3-12B). The P388D.1 macrophages showed a greater reduction in nitrite production at 6 hours after treatment with 1 mM 4-AP, but the cells exhibited a steady recovery to control levels between 12 and 72 hours post-treatment (Fig. 3-12B). A 50 μM concentration of quinine caused a smaller reduction in nitric oxide production than either TEA or 4-AP by 6 hours post-treatment, but, thereafter, there was a steady decrease in nitric oxide production between 12 and 72 hours post-treatment. Examination of the respiration of the P388D.1 macrophages showed that the potassium channel inhibitors had minimal effects on the viability of the macrophages: a slight decrease in respiration at 72 hours for TEA, a slight but steady increase in respiration for 4-AP, and no effect on respiration for quinine (data not shown).

Treatment of the B10-4(S) macrophages with 10 mM TEA, 1 mM 4-AP, and 50 μM

quinine had a much more pronounced effect on their nitric oxide production and viability than the same treatments had on the P388D.1 macrophages (Fig. 3-13). TEA caused a significant reduction in nitric oxide production, compared to control, by 12 hours of incubation (there was no detectable nitrite at 6 hours), followed by partial recovery at 24 hours, and then a further decrease in nitric oxide response from 48 hours to 72 hours post-treatment (Fig. 3-13B). Treatment of the B10-4(S) macrophages with 4-AP abolished detectable nitric oxide production for the first 12 hours of incubation but, like the P388D.1 macrophages, with longer incubations there was some recovery of nitric oxide production (Fig. 3-13B). Quinine caused a large reduction in nitric oxide production by 12 hours post-treatment and longer incubation caused a further decrease until 48 hours (Fig. 3-13B). The viability of the B10-4(S) macrophages was not affected by the potassium channel inhibitor incubations (data not shown).

Effects of Activator Pre-treatments on the NO Production of Potassium Channel Inhibitor-Treated Macrophages

The effects of 6 hour pre-treatments, with various activators and inhibitors, on the nitric oxide production of the macrophage cell lines treated for a further 24 hours with potassium channel inhibitors were assessed to determine which stage of activation was being affected by the potassium channel inhibitors. The control treatments (Figs. 3-14A and 3-15A) were the nitric oxide productions of B10-4(S) and P388D.1 cells that had only been treated with 1 μg/mL LPS and 25 U/mL IFN-γ in the various pre-treatment plates. For the B10-4(S) cell line, a 6 hour pre-treatment with 25 U/mL IFN-γ caused a significant ($p<0.05$) increase in the nitric oxide production compared to the DMEM pre-treatment cultures, whereas pre-treatment with 1 μg/mL LPS or 1 μg/mL LPS and 25 U/mL IFN-γ surprisingly caused a significant ($p<0.05$) reduction in the nitric oxide production compared to the DMEM pre-treatment cultures (Fig. 3-14A). This may have been because

the pre-treatment with LPS induced LPS-desensitization in the B10-4(S) cells that lowered the total nitric oxide production of the cells. For the inhibitor pre-treatment plate, the control's nitric oxide production was significantly higher than the DMEM pre-treatment cultures (Fig 3-14A). Treating the activated B10-4(S) cells with 10 mM TEA, 1 mM 4-AP, and 50 μM quinine produced the expected reductions in the nitric oxide of the DMEM pre-treatment cultures (Fig. 3-14B). Pre-treating the B10-4(S) cells with 10 mM TEA, 1 mM 4-AP, and 50 μM quinine (the Inhibitor pre-treatment plate) caused significantly ($p<0.05$) greater reductions in nitric oxide production than those in the DMEM pre-treatment plate (Fig. 3-14B). In contrast, 6 hour pre-treatments with IFN-γ or LPS and IFN-γ partially restored the nitric oxide production of macrophages treated with the potassium channel inhibitors, with the LPS and IFN-γ pre-treatment being most effective at restoring nitric oxide production (Fig. 3-14B). Pre-treating the B10-4(S) cells with LPS alone was consistently less effective at restoring the nitric oxide production than the other activator pre-treatments (Fig. 3-14B). In fact, the LPS pre-treated B10-4(S) cells that were treated with 10 mM TEA had a significantly lower nitric oxide production than the DMEM pre-treatment cultures (Fig. 3-14B).

For the P388D.1 cell line, a 6 hour pre-treatment with 25 U/mL IFN-γ, 1 μg/mL LPS, or 25 U/mL IFN-γ and 1 μg/mL LPS significantly ($p<0.05$) increased the nitric oxide production compared to the DMEM pre-treatment cultures (Fig. 3-15A). The IFN-γ and LPS pre-treatment produced the greatest enhancement of nitric oxide production with the IFN-γ pre-treatment causing the next greatest enhancement and the LPS pre-treatment producing the smallest enhancement of nitric oxide production (Fig. 3-15A). The inhibitor pre-treatment cultures had the same nitric oxide production as the DMEM control cultures (Fig. 3-15A). Activated P388D.1 cells treated with 10 mM TEA, 1 mM 4-AP, and 50 μM quinine had the expected reductions in nitric oxide production in the DMEM pre-treatment

cultures (Fig. 3-15B). Pre-treating the P388D.1 cells with 10 mM TEA, 1 mM 4-AP, and 50 μM quinine (the Inhibitor pre-treatment plate) caused significantly ($p<0.05$) greater reductions than those in the DMEM pre-treatment cultures (Fig. 3-15B). In contrast, 6 hour pre-treatments with IFN-γ, LPS, or LPS and IFN-γ usually partially restored the nitric oxide production of macrophages treated with 10 mM TEA, 1 mM 4-AP, and 50 μM quinine (Fig. 3-15B). The LPS and IFN-γ pre-treatment was most effective at restoring nitric oxide production, followed by the IFN-γ only pre-treatment, and then the LPS only pre-treatment (Fig. 3-15B). For the 50 μM quinine, only the LPS and IFN-γ pre-treatment restored the nitric oxide production of the inhibitor-treated P388D.1 cells (Fig. 3-15B)

Discussion

The dose-dependent reductions in the nitric oxide production of the B10-4(S) and P388D.1 cell lines induced by treating activated macrophages with the potassium channel inhibitors tetraethylammonium (TEA), 4-aminopyridine (4-AP), and quinine shows that potassium channel function is involved in NO production. In a different study using an inhibitor of ATP-sensitive potassium channels, glibenclamide, Wu *et al.* (1995) concluded that while glibenclamide reduced the nitric oxide production of the macrophage-like J774.2 cells it was not through specific effects on the potassium channels of the macrophages. Rather, they felt the observed reduction in nitric oxide production was due to the secondary effects of glibenclamide on the induction of the inducible nitric oxide synthase enzyme (iNOS) because a general inhibitor of potassium channels, TEA, did not produce reductions in nitric oxide production of the J774.2 cells (Wu *et al.*, 1995). My results do not support this conclusion. By using a number of general inhibitors of potassium channels, we have established that the reduction in nitric oxide production by the inhibitors is due to an effect on macrophage potassium channels. It is highly unlikely that these three structurally-distinct pharmacological agents, in addition to inhibiting the function of

potassium channels, also have the same secondary effect on the induction of iNOS expression. In my study, I observed variations in the sensitivity of the B10-4(S) and P388D.1 cell lines to the different potassium channel inhibitors, especially to TEA. TEA caused a minimal reduction in the nitric oxide production of B10-4(S) cells but caused reductions equivalent to those for 4-AP and quinine in the P388D.1 cells. The inability of TEA to reduce the nitric oxide response in the Wu *et al.* (1995) study may have been because the J774.2 cell line was unresponsive to treatment with TEA.

Treating the B10-4(S) and P388D.1 cell lines with cobalt chloride produced highly unexpected results. The cobalt ion (Co^{2+}) is typically viewed as an inorganic inhibitor of calcium channels that competes with calcium ions (Ca^{2+}) for passage through the pore thereby preventing calcium currents through these channels (Hille, 1992). Since voltage-dependent calcium channels have never been observed in macrophages (Gallin, 1991), cobalt chloride was not expected to alter nitric oxide production, much less produce a biphasic response. However, it was found that ion channel inhibitors classically viewed as blocking only calcium channels also inhibited the currents of both voltage- and calcium-activated potassium channels in a poorly understood manner (Grinstein and Dixon, 1989). Among these inhibitors were the inorganic ions La^{3+}, Ni^{2+}, and Cd^{2+} whose larger size than K^+ probably physically blocked the potassium channel pore. Presumably Co^{2+} would also inhibit potassium channels in the same manner, and this could explain why cobalt chloride caused an inhibition of the nitric oxide production whereas other organic inhibitors of calcium channels, such as verapamil, did not affect the nitric oxide production of macrophages.

This putative ability of Co^{2+} to block many types of ion channels, particularly calcium and potassium channels, may explain the biphasic nitric oxide response seen with cobalt chloride treatment. The Co^{2+} ion may have affected calcium channels at the lower concentrations, resulting in enhancement of the signals or conditions required for nitric

oxide production. At higher concentrations the cobalt chloride may have started to inhibit potassium channels causing decreased nitric oxide production similar to that seen with the other potassium channel inhibitors. Alternatively, the Co^{2+} may actually cause an enhancement of nitric oxide production, but the cytotoxicity associated with cobalt chloride treatment at higher concentrations may indirectly cause lower nitric oxide levels. However, it does not appear that alterations in the nitric oxide production of the macrophage cell lines can be directly related to changes in the cell respiration. This was particularly evident in the P388D.1 cell line treated with cobalt chloride where the cell respiration of the 50, 100, and 250 μM concentrations were not significantly different from one another even though the nitric oxide production at each of the concentrations varied significantly from one to another. This clearly indicates that an ion channel inhibitor could affect an aspect of the metabolism of cytotoxic activation that was not related to the viability of the macrophages. Therefore, although the cytotoxicity of cobalt chloride was probably responsible for a part of the reduced nitric oxide production of the macrophage cell lines, it seems likely that a part of the biphasic alterations in the nitric oxide production was due to cobalt's ability to affect both calcium channels and potassium channels.

In contradiction to the reductions in the viability of both B10-4 and P388D.1 cells seen in the XTT viability experiments (Appendix 1), TEA, 4-AP, and quinine did not cause reductions in the cell respiration of the macrophages at the same concentration ranges in this set of experiments. The most probable explanation for this maintenance of XTT viability is that the potassium channel inhibitors did not disrupt cell metabolism or cause cell death until 48 hours of incubation or later. For example, from the XTT viability experiments it was seen that all TEA concentrations had equivalent cell respiration values at 24 hours, but by 48 hours the higher TEA concentrations began to have negative effects on the cell respiration (Appendix 1). The reduction in respiration of the macrophages caused by TEA at the longer incubation periods may explain part of the reduction in nitric oxide production

because there would be fewer cells to produce nitric oxide. This reduction in respiration may be due to potassium channel inhibitors causing non-specific toxicity of the cells. Alternatively, the loss of ion homeostasis within the cells as the potassium channels are blocked from functioning properly may be causing cell death. Nevertheless, TEA caused a significant reduction in the nitric oxide production at 12 and 24 hours post-treatment prior to the times when the respiration of the cells was being affected. Furthermore, the general ineffectiveness of quinine and 4-AP to reduce the respiration of the macrophages at any incubation period, while causing large reductions in the nitric oxide production, suggests that the reduction in nitric oxide production is not simply due to toxic effects of the potassium channel inhibitors on the cell lines. The blockage of potassium channel inhibitors must in some way be involved in the induction or production of nitric oxide by iNOS. Our results show that treatment of activated macrophages with an agonist of potassium channels significantly increased the nitric oxide production of the cells indicating that the alterations in the function of potassium channels can cause significant changes in the nitric oxide production of activated macrophages. If the observed effects of potassium channel inhibitors on nitric oxide production were due only to non-specific toxicity that reduced either cell number or individual cell viability then a potassium channel agonist would not be expected to enhance nitric oxide production.

Potassium channels could be involved in the intracellular signals associated with either the priming stage inducing formation of iNOS or the triggering step activating iNOS. Theoretically, the potassium channels could be responsible for producing conditions necessary for the iNOS enzyme to function at maximum efficiency. However, Wu *et al.* (1995) convincingly showed that glibenclamide, an inhibitor of ATP-sensitive potassium channels, does not affect the function of iNOS. Glibenclamide was less effective at inhibiting the nitric oxide production of J774.2 cells when presented several hours after LPS activation compared to when presented with LPS, whereas the iNOS inhibitor L-

NAME (N^{ω}-nitro-L-arginine methyl ester) was equally effective at inhibiting nitric oxide production when presented from 2 to 10 hours after treating cells with LPS (Wu *et al.*, 1995). Therefore, the reductions in nitric oxide production was not due to a reduction in iNOS function suggesting that potassium channels must be involved in inducing the formation and activation of iNOS.

Macrophages possess a number of types of voltage-gated potassium channels that the inhibitors may be affecting. Researchers using patch clamp and whole cell voltage clamp techniques have found delayed outward rectifier, calcium-dependent, ATP-dependent, and inward rectifier potassium channels within macrophages (Randriamampita and Trautmann, 1987; Gallin, 1991; Hara *et al.*, 1991; MacKinnon, 1991; Wu *et al.*, 1995). It might be that a single type of potassium channel is directly responsible for the reduced nitric oxide response. However, glibenclamide only affects ATP-sensitive potassium channels and 4-AP only affects the delayed outward rectifier potassium channels of macrophages, yet both inhibitors are effective at reducing nitric oxide production of macrophages (Gallin, 1991; Hille, 1992; Wu *et al.*, 1995). Therefore, it is unlikely that the reduction of the nitric oxide response of macrophages caused by the potassium channel inhibitors is due to blockade of a single potassium channel type.

The potassium channel inhibitors used in our study affect numerous types of potassium channels and affect those channel types through different mechanisms. In our experimental set-up, TEA was acting from outside the cell by binding to a specific receptor site on the extracellular side of the potassium channel and physically blocking the pore (Cook and Quast, 1990; Robertson and Steinberg, 1990; Hille, 1992; Jan and Jan, 1992). Delayed-rectifier, inward rectifier, calcium-sensitive and ATP-sensitive potassium channels are all affected by TEA, with only the potency of TEA required to achieve half-maximal blockage varying from potassium channel type to potassium channel type (Cook and Quast, 1990;

Robertson and Steinberg, 1990; Hille, 1992). The inhibitor 4-AP is lipid soluble and membrane permeant, allowing it to cross cell-membranes to reach its site of action (Cook and Quast, 1990; Robertson and Steinberg, 1990; Hille, 1992). The binding site for 4-AP is probably within the pore of the potassium channel, and 4-AP likely binds the channel by either directly diffusing out of the cell membrane or diffusing from the cytoplasmic side of the channel once the physical gate of the potassium channel opens (Cook and Quast, 1990; Robertson and Steinberg, 1990; Hille, 1992). Within macrophages, 4-AP physically blocks the pore of the delayed rectifier type potassium channels. The alkaloids quinine and quinidine are stereoisomers isolated from plants of the *Cinchona* family that presumably block potassium channels through a similar mechanism (Cook and Haylett, 1985; Cook and Quast, 1990; Natick, 1997). Quinidine, like 4-AP, is a membrane-permeant molecule that blocks potassium channels by passing into the cytoplasm of cells or diffusing from the cell membrane to bind the receptor within the pore and physically block the passage of K^+ ions (Cook and Quast, 1990; Robertson and Steinberg, 1990; Hille, 1992). Quinidine and quinine are equally potent at blocking delayed rectifier, calcium-sensitive, and ATP-sensitive potassium channels of macrophages (Cook and Quast, 1990). The exact concentration required for half-maximal inhibition of any particular potassium channel varies from type to type (Cook and Quast, 1990; Robertson and Steinberg, 1990; Hille, 1992). Given the wide variety of potassium channel types being affected by the potassium channel inhibitors, it seems likely that each potassium channel type contributes a small amount to the activation or signaling process responsible for inducing nitric oxide production probably by helping establish a specific membrane potential. When that contribution is blocked by a particular inhibitor, as shown in this study, it affected the nitric oxide production of the macrophages through a poorly understood process.

The order of activator and inhibitor treatments was examined to establish whether potassium channels were involved in the priming stage or the triggering stage of

macrophage activation. By pre-priming the macrophages with IFN-γ or pre-triggering the macrophages with LPS prior to inhibition of the macrophage's potassium channels, it was possible to determine if the potassium channels were important in the priming or triggering stage of activation. A 6 hour pre-treatment of the B10-4(S) and P388D.1 cells with the potassium channel inhibitors prior to activation with IFN-γ and LPS caused a greater reduction in the nitric oxide production of both cell lines compared to cells undergoing a 6 hour pre-incubation in DMEM. Conversely, 6 hour pre-treatments with IFN-γ or IFN-γ and LPS resulted in significant restorations of nitric oxide production in macrophages treated with 10 mM TEA, 1 mM 4-AP, and 50 μM quinine. Pre-treatment with LPS alone usually partially restored the nitric oxide production, but LPS was far less effective at restoring nitric oxide production than either IFN-γ alone or IFN-γ with LPS. In fact, LPS did not cause a restoration of nitric oxide production of 50 μM quinine treated P388D.1 cells, and it caused a significantly greater reduction than the DMEM pre-treatment in the nitric oxide production of 10 mM TEA treated B10-4(S) cells. Pre-treatment with LPS and IFN-γ together was most effective at restoring the nitric oxide production of potassium channel inhibitor treated macrophages, but pre-treatment with IFN-γ alone was only slightly less effective. Since pre-treatment with LPS consistently either did not restore the nitric oxide production of potassium channel inhibitor treated macrophages or induced a lower restoration than IFN-γ, it seems likely that potassium channels are involved in the priming stage of macrophage activation because treatment with IFN-γ alone primes the macrophages for antimicrobial activity.

A 6 hour priming of the macrophages by IFN-γ or IFN-γ and LPS may have allowed the essential processes or signals that initiates nitric oxide production to occur prior to blockade by the potassium channel inhibitors. Potassium channels are unlikely to be involved in the translation or activation process of iNOS because by 6 hours following

activator treatment, macrophages have already transcribed large amounts of iNOS mRNA (Xie and Nathan, 1994; Wu *et al.*, 1995). If it were the translation or activation processes that were being affected by alteration in the potassium channel activity it would be expected that IFN-γ or LPS pre-treated cells would have the same reduction in nitric oxide production as the DMEM pre-treatment because the iNOS mRNA transcripts would still have to be translated or activated and as such could still be affected by the later treatment with the potassium channel inhibitors. Since activator pre-treatment significantly restores the nitric oxide production, presumably by giving the cells sufficient time to transcribe iNOS mRNA prior to potassium channel inhibitor treatment, it seems likely that potassium channel inhibitors are involved in the signals leading to the transcription of the iNOS gene or the stabilization of the mRNA transcripts.

In summary, activation or inhibition of potassium channels clearly results in an equivalent effect, whether enhancing or inhibitory, on the nitric oxide production of macrophage-like cell lines. This alteration in nitric oxide production is likely due to effects of altered potassium channel activity on one of the intracellular signaling steps in the cascade that primes the macrophage and induces iNOS. The exact intracellular signaling steps being affected and the mechanism of potassium channel involvement remain to be elucidated.

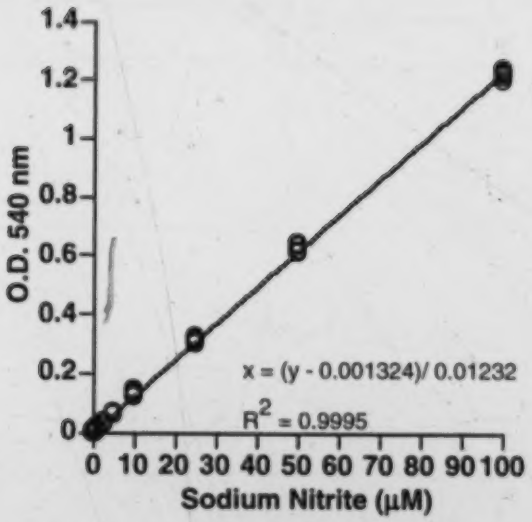

Figure 3-1. Calibration of the NO assay (Griess reaction) with sodium nitrite. The line represents the linear regression relationship between the concentration of sodium nitrite and the change in O.D. 540 nm following the NO assay.

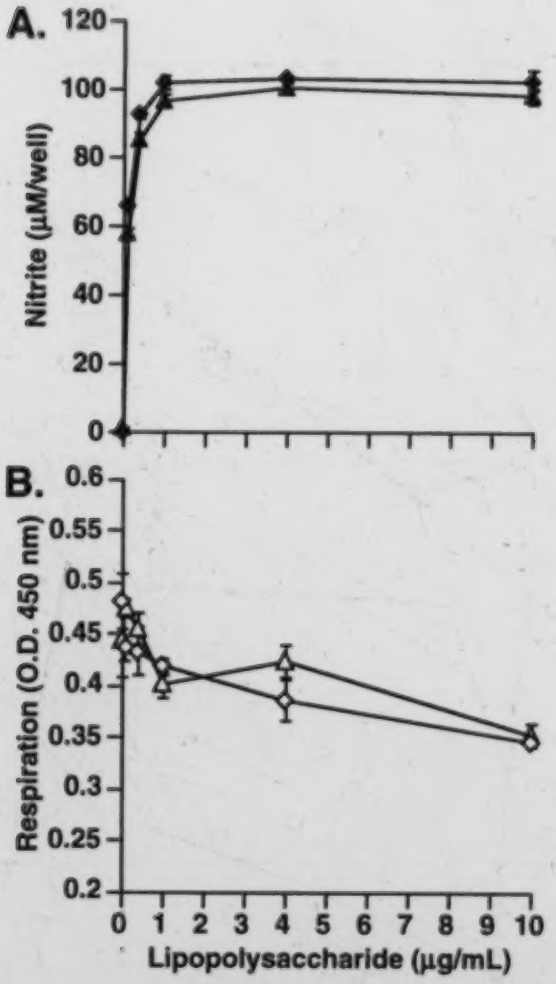

Figure 3-2. LPS dose response of the B10-4(S) cell line. The effects of increasing concentrations of LPS on the NO production (A.) and viability (B.) of B10-4(S) cells, with (◆, ◇) and without (▲, △) 25 U/mL IFN-γ, were examined after a 24 hour incubation.

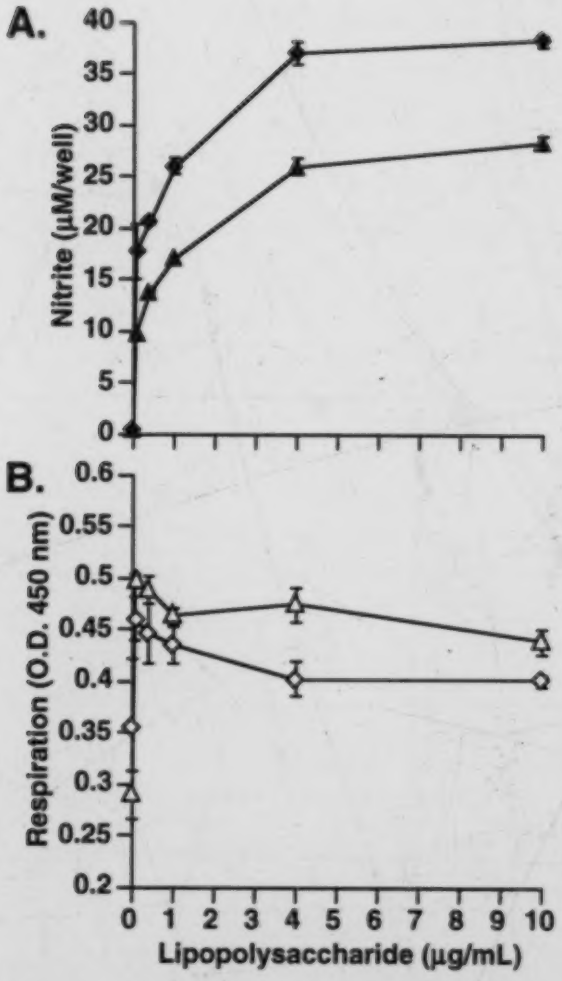

Figure 3-3. LPS dose response of the P388D.1 cell line. The effects of increasing concentrations of LPS on the NO production (A.) and viability (B.) of P388D.1 cells, with (◆, ◇) and without (▲, △) 25 U/mL IFN-γ, were examined after a 24 hour incubation.

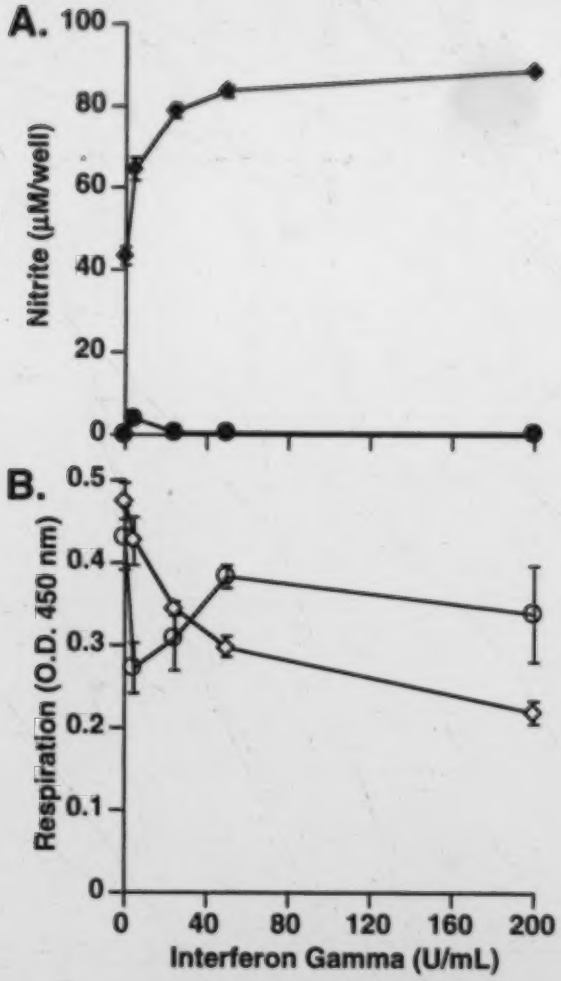

Figure 3-4. IFN-γ dose response of the B10-4(S) cell line. The effects of increasing concentrations of IFN-γ on the NO production (A.) and viability (B.) of B10-4(S) cells, with (◆, ◇) and without (●, O) 500 ng/mL LPS, were examined after a 24 hour incubation.

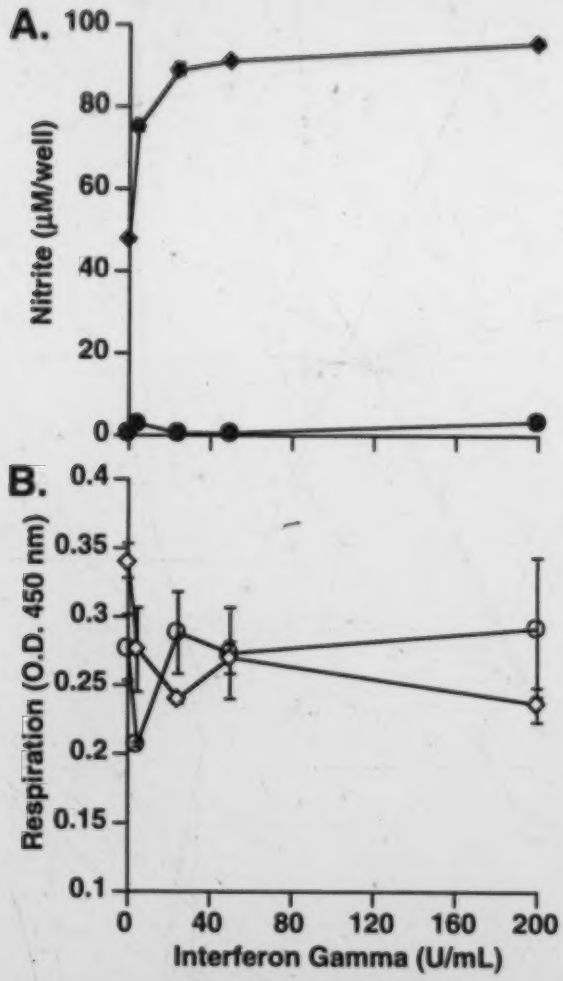

Figure 3-5. IFN-γ dose response of the P388D.1 cell line. The effects of increasing concentrations of IFN-γ on the NO production (A.) and viability (B.) of P388D.1 cells, with (◆, ◇) and without (●, O) 500 ng/mL LPS, were examined after a 24 hour incubation.

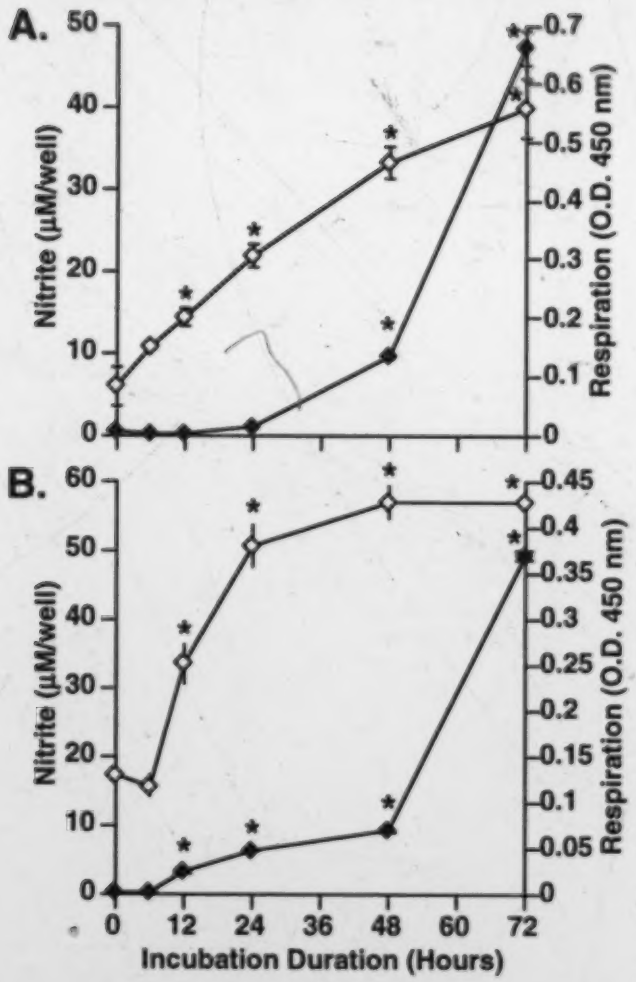

Figure 3-6. Time course of macrophage NO production. The effects of incubation length on the NO production (◆) and viability (◇) of B10-4(S) (A.) and P388D.1 (B.) cells treated with 25 U/mL IFN-γ and 1 µg/mL LPS were examined over a 72 hour period. A * indicates a significant difference from control with $p<0.05$.

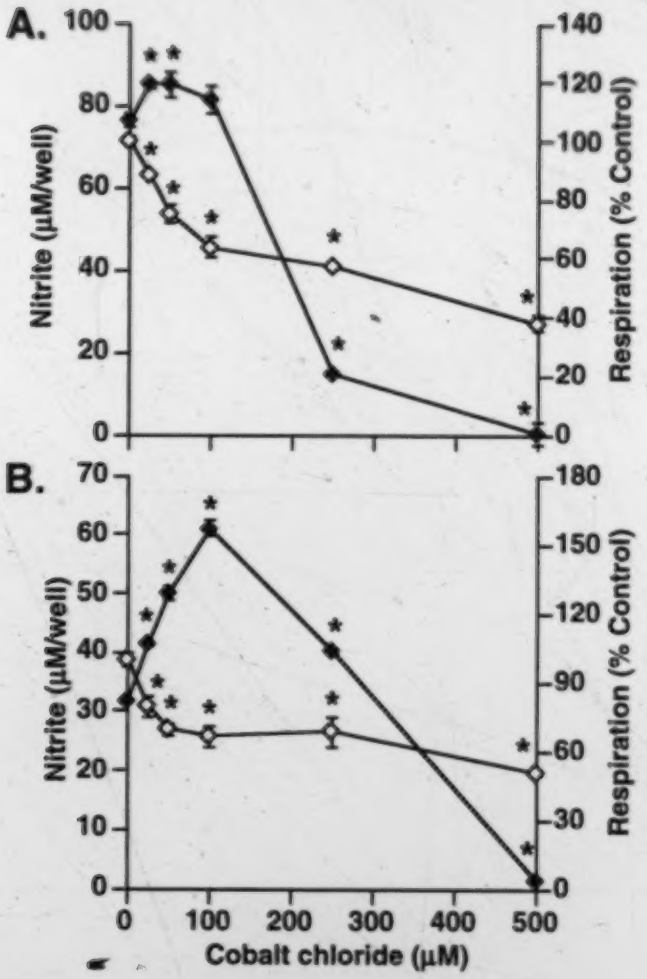

Figure 3-7. Cobalt chloride on macrophage NO production. The effects of increasing cobalt chloride concentration on the NO production (◆) and viability (◇) of B10-4(S) (A.) and P388D.1 (B.) cells treated with 25 U/mL IFN-γ and 1 µg/mL LPS were examined after a 24 hour incubation. A * indicates a significant difference from the 0 µM control with $p<0.05$.

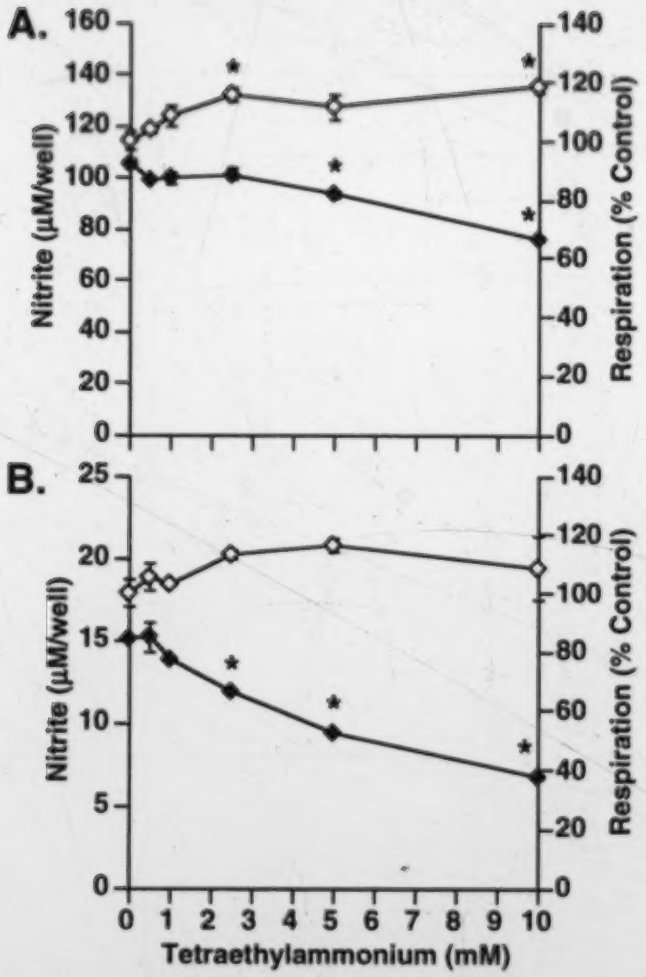

Figure 3-8. Tetraethylammonium on macrophage NO production. The effects of increasing tetraethylammonium concentration on the NO production (◆) and viability (◇) of B10-4(S) (A.) and P388D.1 (B.) cells treated with 25 U/mL IFN-γ and 1 µg/mL LPS were examined after a 24 hour incubation. A * indicates a significant difference from the 0 mM control with $p<0.05$.

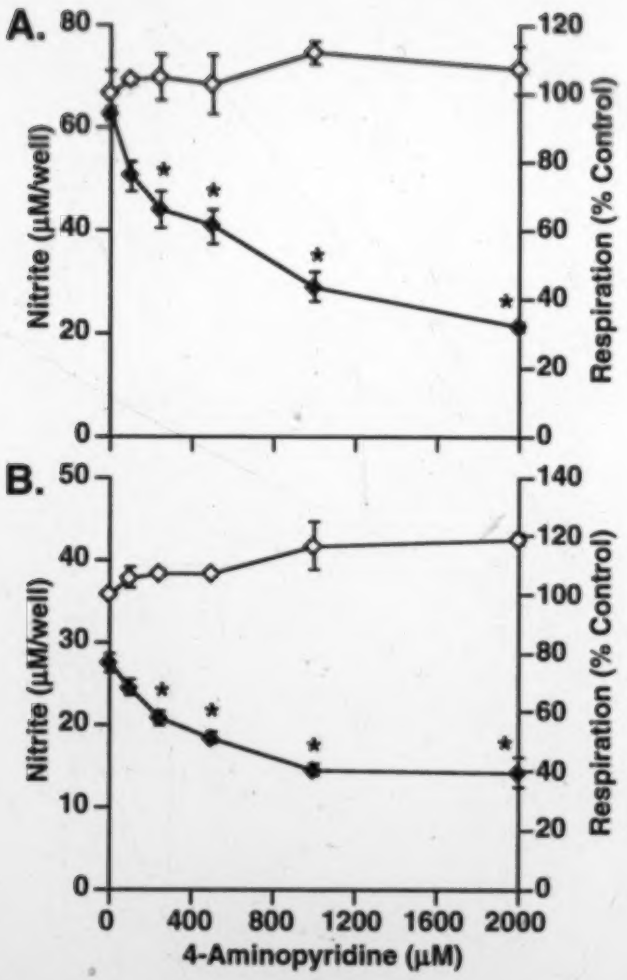

Figure 3-9. 4-Aminopyridine on macrophage NO production. The effects of increasing 4-aminopyridine concentration on the NO production (◆) and viability (◇) of B10-4(S) (A.) and P388D.1 (B.) cells treated with 25 U/mL IFN-γ and 1 µg/mL LPS were examined after a 24 hour incubation. A * indicates a significant difference from the 0 µM control with $p<0.05$.

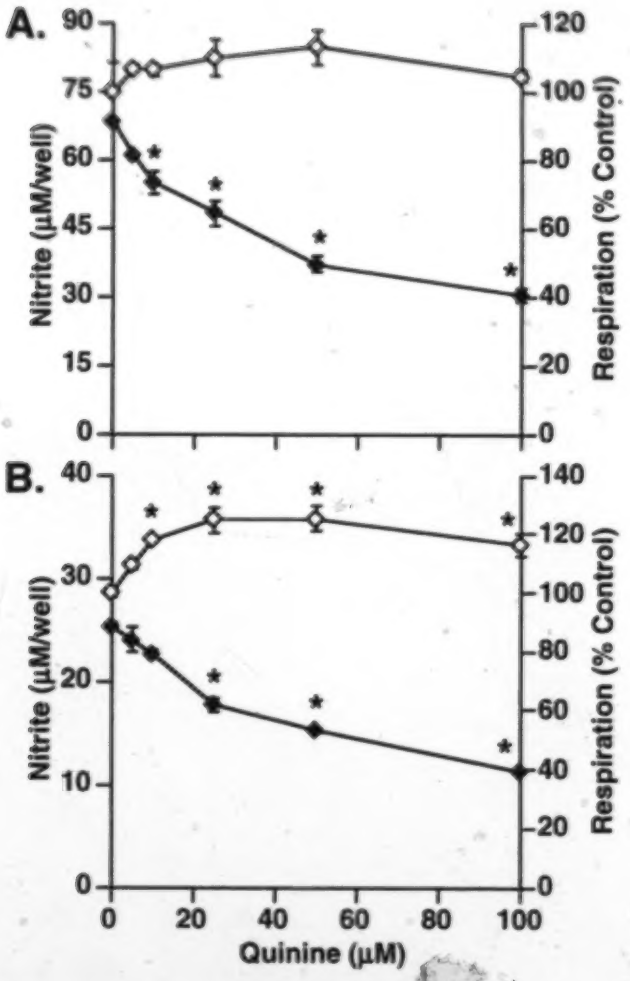

Figure 3-10. Quinine on macrophage NO production. The effects of increasing quinine concentration on the NO production (◆) and viability (◇) of B10-4(S) (A.) and P388D.1 (B.) cells treated with 25 U/mL IFN-γ and 1 µg/mL LPS were examined after a 24 hour incubation. A * indicates a significant difference from the 0 µM control with $p<0.05$.

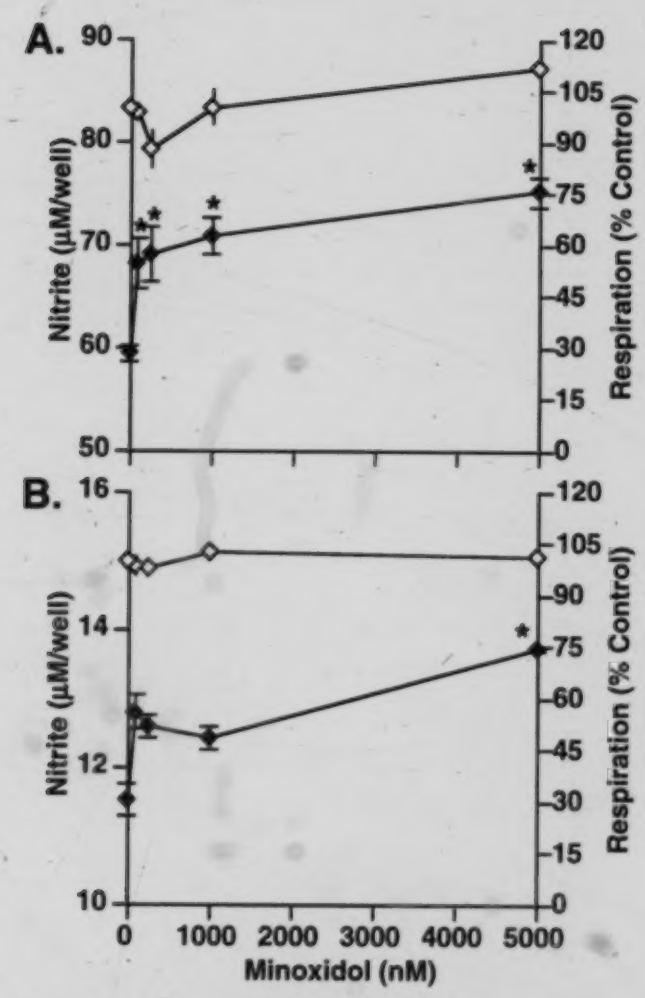

Figure 3-11. Minoxidol on macrophage NO production. The effects of increasing minoxidol concentration on the NO production (◆) and viability (◇) of B10-4(S) (A.) and P388D.1 (B.) cells treated with 25 U/mL IFN-γ and 1 µg/mL LPS were examined after a 24 hour incubation. A * indicates a significant difference from the 0 nM control with $p<0.05$.

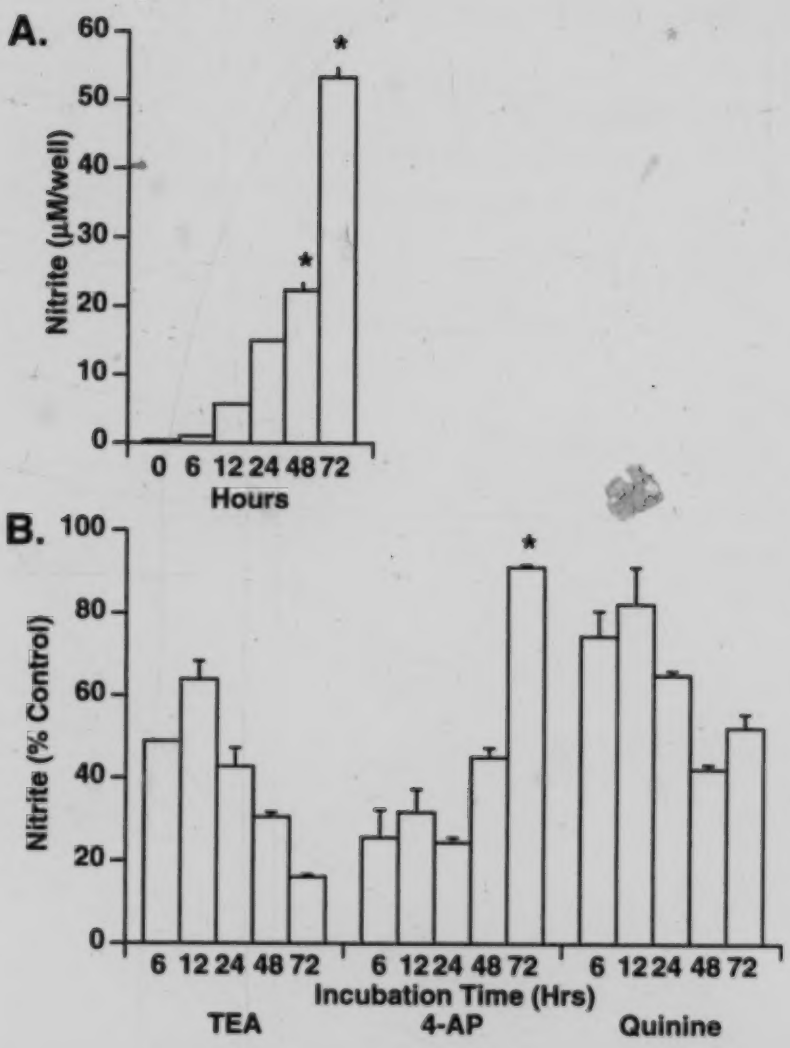

Figure 3-12. Time course of P388D.1 NO production during ion channel inhibitor treatment. The NO production of 25 U/mL IFN-γ/1 µg/mL LPS activated P388D.1 cells treated with complete DMEM (control) (A.) or ion channel inhibitors (10 mM TEA, 1 mM 4-aminopyridine, and 50 µM quinine) (B.) was assessed over a 72 hour incubation period. A * indicates a significant difference from the previous time point at $p<0.05$.

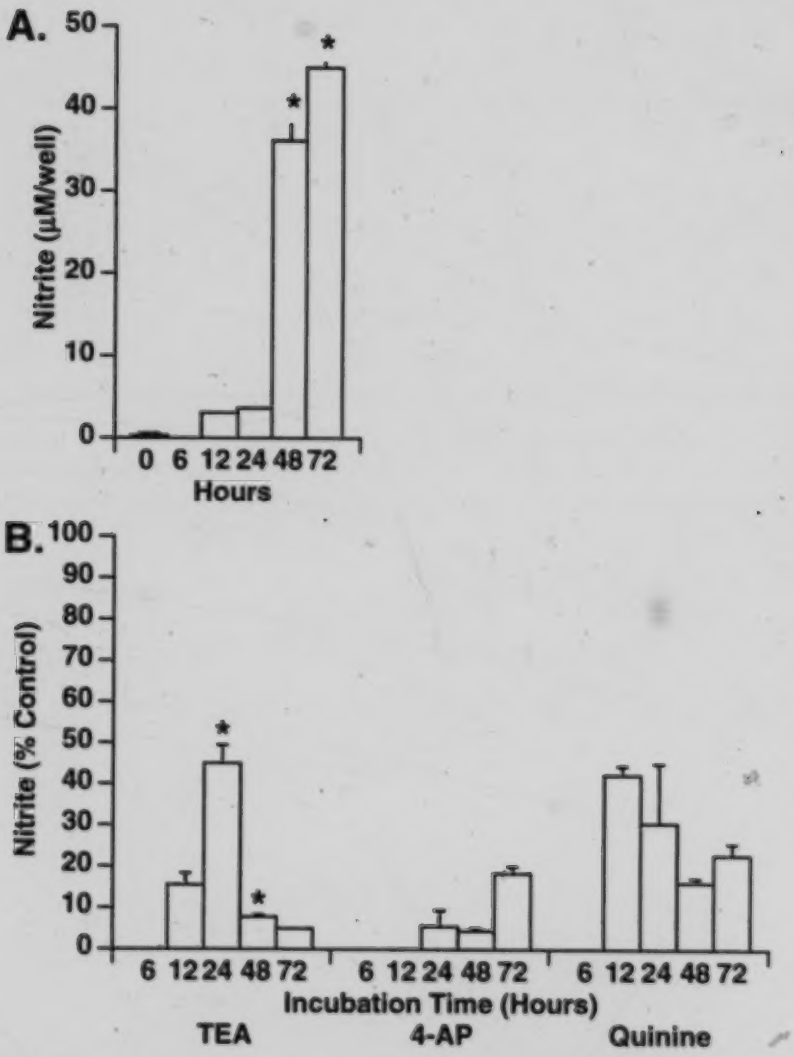

Figure 3-13. Time course of B10-4(S) NO production during ion channel inhibitor treatment. The NO production of 25 U/mL IFN-γ/1 µg/mL LPS activated B10-4(S) cells treated with complete DMEM (control) (A.) or ion channel inhibitors (10 mM TEA, 1 mM 4-aminopyridine, and 50 µM quinine) (B.) was assessed over a 72 hour incubation period. A * indicates a significant difference from the previous time point at $p<0.05$.

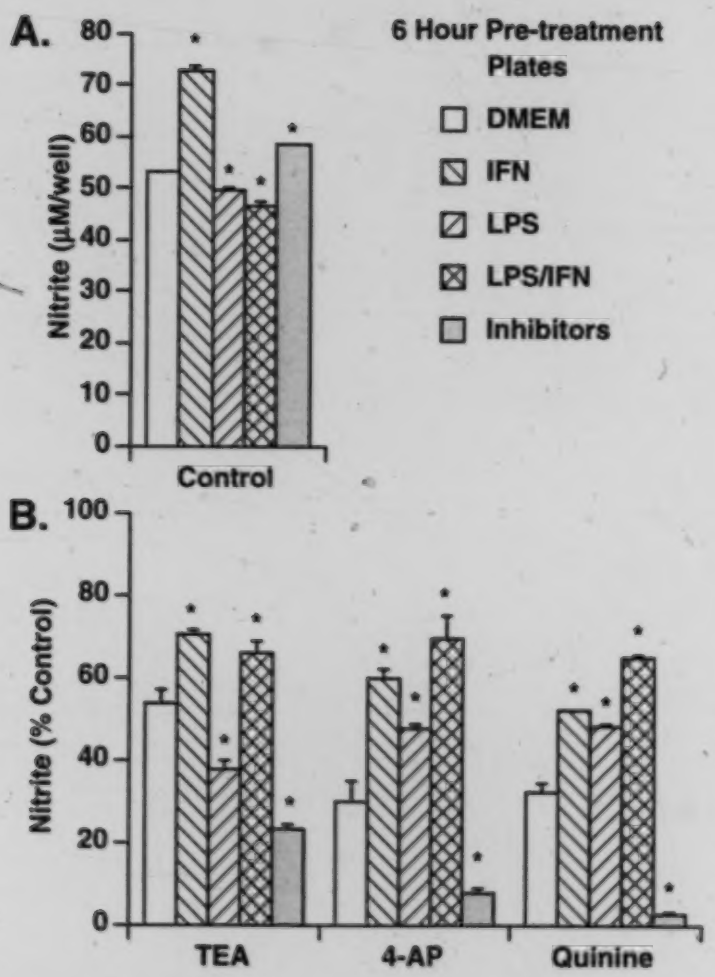

Figure 3-14. Restoration of inhibitor-reduced NO production by activator pre-treatments. The effects of a 6 hour pre-treatment with various activators on the NO production of 25 U/mL IFN-γ/1 µg/mL LPS activated B10-4(S) cells (A.) and 25 U/mL IFN-γ/1 µg/mL LPS activated B10-4(S) cells treated with 10 mM TEA, 1 mM 4-aminopyridine, or 50 µM quinine (B.). A * indicates a significant difference from the DMEM plate at $p<0.05$.

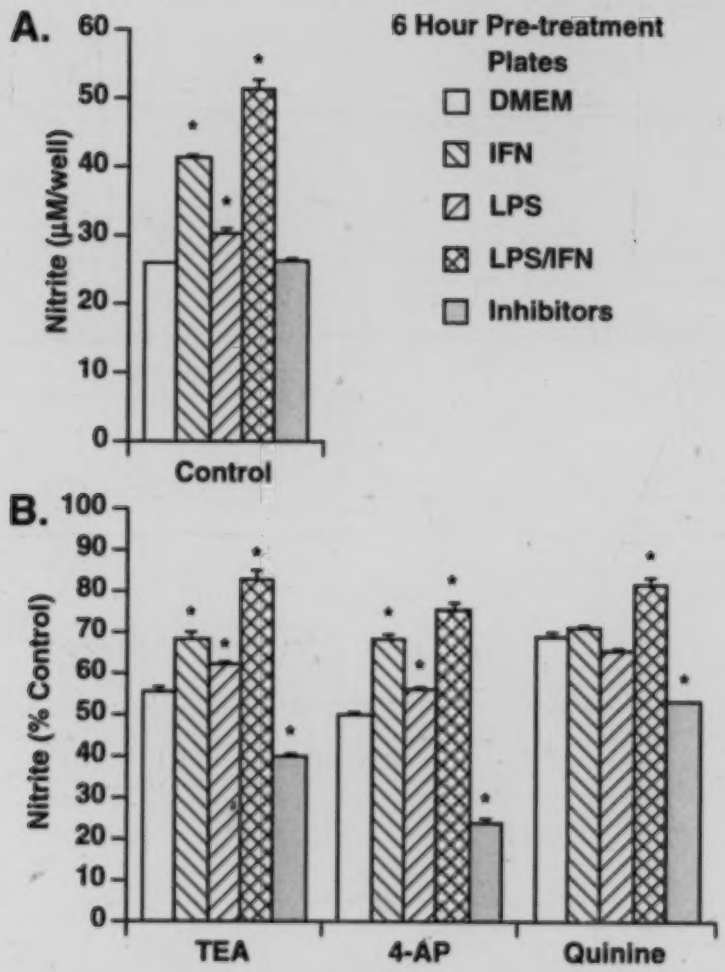

Figure 3-15. Restoration of inhibitor-reduced NO production by activator pre-treatments. The effects of a 6 hour pre-treatment with various activators on the NO production of 25 U/mL IFN-γ/1 µg/mL LPS activated P388D.1 cells (A.) and 25 U/mL IFN-γ/1 µg/mL LPS activated P388D.1 cells treated with 10 mM TEA, 1 mM 4-aminopyridine, or 50 µM quinine (B.). A * indicates a significant difference from the DMEM plate at $p<0.05$.

Chapter 4 : General Discussion

The activation of macrophages for cytotoxic function is a complex process involving multiple intracellular signaling components including G proteins, protein tyrosine kinases, $[Ca^{2+}]_i$ fluxes, protein kinase C, and numerous nuclear factors. Ion channels represent another method for the propagation of the intracellular signaling cascades that cause macrophage activation. The role of calcium channels in the activation process of macrophages appears to be limited to releasing the intracellular calcium stores from the endoplasmic reticulum, Golgi complex, and nucleus. Macrophages do not possess voltage-gated calcium channels in their plasma membranes (Gallin, 1991), but the rapid increase in $[Ca^{2+}]_i$ following treatment with PAF and LPS suggests that second messenger-gated calcium channels exist in the membranes of the organelles that store calcium (Randriamampita and Trautmann, 1989; Letari *et al.*, 1991b; Katnik and Nelson, 1993). Although these second-messenger gated calcium channels release calcium into the cytoplasm to activate a variety of calcium-dependent processes and enzymes, the buffered reduction in $[Ca^{2+}]_i$ immediately following an increase in $[Ca^{2+}]_i$ is caused by the activation of slower Ca^{2+}-ATPases that re-store released Ca^{2+} (Jun *et al.*, 1996). Presumably, it was the activity of such calcium buffering processes and enzymes that resulted in the reduced $[Ca^{2+}]_i$ of the macrophages observed from one to 24 hours after treatment with LPS (Chapter 2). Treatment with LPS probably caused an increase in $[Ca^{2+}]_i$ similar to that seen by Letari *et al.* (1991b) and Raddassi *et al.* (1994), whether or not it was induced by cross-reactivity with the PAF receptor, and this increase likely induced buffering of $[Ca^{2+}]_i$ to levels below those of resting macrophages. The reduction of $[Ca^{2+}]_i$ caused by 1 µg/mL LPS was initiated prior to and persisted long after induction of iNOS metabolic machinery at 6-8 hours post-treatment (Steuhr and Marletta, 1987). This suggests that rather than purely being a by-product of an LPS-induced increase in $[Ca^{2+}]_i$, the reduced $[Ca^{2+}]_i$ may

play a physiological role in the macrophage activation process. The lowered $[Ca^{2+}]_i$ could either inactivate calcium-dependent signals that control aspects of metabolism that are not required in the activated macrophage or activate aspects of metabolism that are required in the activated macrophage by removing the effects of repressor enzymes. Therefore, although the role of calcium channels in macrophage activation appears to be limited to the release of intracellular calcium stores, the calcium ion itself probably plays a major role in regulating the activity of signaling processes that controls the metabolism of the macrophage.

Potassium channels may play an exciting and more direct role in the propagation of intracellular signaling cascades that are responsible for priming macrophages for cytotoxic function. Treating 1 μg/mL LPS and 25 U/mL IFN-γ activated macrophage-like B10-4(S) and P388D.1 cell lines with potassium channel inhibitors caused dose-dependent reductions in the nitric oxide production of both cell lines. Since potassium channel inhibitors do not directly affect the function of the iNOS enzyme (Wu *et al.*, 1995) and since the amount of nitric oxide produced by activated macrophages is directly proportional to the amount of iNOS mRNA transcribed (Weisz *et al.*, 1994), potassium channels must either be responsible for producing intracellular conditions needed for upregulating expression of the iNOS gene or be directly involved in the upregulation itself. The activity of the iNOS enzyme is not affected by modulating proteins such as calmodulin or by kinase- or phosphatase-induced covalent modification (Xie and Nathan, 1994), so the primary way that intracellular signals can modify iNOS activity is by regulating the transcription of the gene. Potassium channels provide the largest contribution in maintaining the membrane potential of macrophages (Gallin, 1991), and alterations in their activity changes the membrane potential of the cell (Eckert *et al.*, 1988; Hille, 1992). By maintaining the membrane potential around a specific value, potassium channels could be establishing conditions that allow many signals to occur. Alternatively, the potassium

channels may actively participate in intracellular signaling cascades by regulating the intracellular concentration of potassium ions to cause alterations in membrane potential.

Recent research has shown that ion channels could theoretically be integrated into the intracellular signaling cascades responsible for activation of macrophages. The activity of ion channels can be regulated by covalent modification of residues in their protein subunits by G proteins, providing a method for intracellular signaling cascades to intersect with ion channels (McKinney and Gallin, 1992; Wickman and Clapham, 1995). Treating cells of the J774.1 macrophage-like cell line or murine primary bone marrow derived macrophages intracellularly with the G protein activators guanosine-5'-*o*-thiotriphosphate (GTPγS), or 5'-guanylylimidodiphosphate (GppNHp) induced a distinct outwardly rectifying potassium current that was measured using the whole-cell patch clamp technique (McKinney and Gallin, 1992). The exact mechanism by which G proteins recruit potassium channels was uncertain, but it was probably through a covalent modification of amino acid residues in the potassium channels because treatment with the second messenger molecules cAMP and IP_3 normally produced by G proteins did not induce the potassium current (McKinney and Gallin, 1992). Once the potassium channels are activated the intracellular signals may then be propagated through effects on the membrane potential of the cell.

Potassium channels provide the largest contribution of all the ion channels to establishing the membrane potential of cells (Hille, 1992), and inhibiting the function of potassium channels alters the membrane potential of the cells (Gallin, 1991; Haslberger *et al.*, 1992). Such alterations of the membrane potential have been shown to have direct effects on cytotoxic functions of macrophages, such as post-transcriptionally interfering with the LPS-induced secretion of TNF-α (Haslberger *et al.*, 1992),. More importantly, however, alterations in membrane potential also affect the enzymatic function of intracellular signaling proteins. Artalejo *et al.* (1992) determined that the activation of an

unknown protein kinase was dependent on the membrane potential of chromaffin cells because either the substrate or the kinase itself had a voltage-dependent subunit that was triggered by the alteration in the membrane potential to produce a conformation that allowed the phosphorylation to proceed. This provides a clear method for intracellular signals to be indirectly propagated by potassium channels, and the potential amplification of a signal passing through potassium channels would be enormous since any process in the cell with a charge sensitive subunit, whether an intracellular signaling protein or a metabolic enzyme, would be affected.

The involvement of calcium channels in the cytotoxic activation of leukocytes has long been recognized because of Ca^{2+} ion's direct role as a second messenger molecule (Scharff and Foder, 1993). However, recognition that other ion channels, particularly potassium channels, play roles in leukocyte activation is slow in coming. For many types of leukocytes, connections between potassium channels and the ability to perform cytotoxic functions have been detected as abrogations of cytotoxic function following treatment with potassium channel inhibitors (Schlicter *et al.*, 1986; Eleno *et al.*, 1990; Haslberger *et al.*, 1992; Wu *et al.*, 1995). Yet, even with such evidence of the involvement of potassium channels in cytotoxic activation, almost no one has proposed a role for potassium channels, or other non-excitable ion channels, in the intracellular signaling cascades that induce leukocyte activation. The non-excitable ion channels (potassium and chloride channels) have long been thought to only play roles in homeostasis of the cell by maintaining cell volume and membrane potential without having roles in activation processes (Gallin, 1991). This does not appear to be the actual situation. Considering the many methods of regulating the activity of ion channels (voltage-gating, ligand-gating, second messenger-gating, or some combination of the above), and the widespread effects throughout the entire cell that changes in ion channel activity can have, it should not be surprising for a role in intracellular signaling to occur. Important enzymes or processes that have voltage-

dependencies include all types of voltage-gated ion channels, kinases, and the ribosomes responsible for translation of mRNA transcripts, and more will undoubtedly be found as researchers look for them (Artalejo *et al.*, 1992; Haslberger *et al.*, 1992; Hille, 1992).

Ion channels, and particularly potassium channels, appear to be good candidates for propagating intracellular signals. Series of signaling cascades could be initiated by the cell assuming a specific membrane potential that activates different putative voltage-sensitive enzymes set to that particular potential. All that remains is to clearly establish a connection between potassium channels and the intracellular signaling complexes that allows signals to be propagated through potassium channels.

Future Research

The kinetics of the decrease in intracellular calcium concentration caused by cytotoxic activation of macrophages must be examined to determine exactly when the changes occur and how long they persist. Furthermore, the effects of the lowered calcium concentration on metabolic processes within the macrophage should be examined to determine if the reduction is truly just a byproduct of a transient increase in $[Ca^{2+}]_i$ or if it plays an actual physiological role in macrophages. It would also be interesting to determine the effects on the $[Ca^{2+}]_i$ of infecting macrophages with an obligate intracellular parasite such as *Leishmania major* to determine if the parasite interferes with alterations in $[Ca^{2+}]_i$ associated with macrophage activation. Obligate intracellular parasites employ a variety of methods to avoid the antimicrobial functions of macrophages, including interfering with the propagation of activating signals (Reiner 1994; Olivier *et al.*, 1992). By preventing changes in $[Ca^{2+}]_i$ *Leishmania* may prolong its survival in macrophages.

The involvement of potassium channels in the intracellular signals activating macrophages must be more fully explored. A connection between the two has indirectly been shown to exist in this thesis; however, future studies should directly examine the effects of altering potassium channel activity on specific intracellular signaling components

involved in the induction of nitric oxide production in macrophages to further characterize the mechanism of involvement and the stage of activation being affected. Good intracellular signaling candidates for future studies are the production of inositol triphosphate, the production of diacylglycerol, and the activation of protein kinase C. In addition, studies should be undertaken to determine if it is alterations in membrane potential resulting from altered activity of potassium channels that cause changes in the nitric oxide production of the macrophages. Work in this area has the potential to elucidate a fascinating and new method that cells may use to transduce signals from the extracellular milieu into the cell.

Finally, it would be interesting to determine the effects of treating *Leishmania*-infected macrophages with potassium channel inhibitors to establish a role of potassium channels in the ability of the macrophage to eliminate parasites. Since nitric oxide is such an important effector molecule in the resolution of obligate intracellular parasites, such as *Leishmania major*, the parasites may target the potassium channels as a method of preventing induction of nitric oxide production, and treatment with potassium channel inhibitors may mimic this allowing the parasites to survive.

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Appendix 1 : Validation of the XTT Assay

Introduction

Prior to establishing the effects of any treatment on an aspect of cellular metabolism, the possible toxic effects of such a treatment on the cells being studied must be assessed to ensure that the observed effects are not caused by cell death. Classically, determination of treatment cytotoxicity has been made by counting the number of cells in control and treatment groups (Abbas *et al.*, 1994). However, when many treatments or many doses of each treatment are being employed in an experiment, cell counting can be very time consuming, labour-intensive, and prone to enumeration errors. An efficient alternative method for determining cell viability is through the use of colorimetric assays that monitor changes in the optical density or absorbance at specific wavelengths as reagents react with components of cellular metabolism (Stevens *et al.*, 1991). These indirect measures of cell number or cellular metabolism allow the cytotoxic effects of large numbers of treatments to be assessed in shorter time periods and with greater sensitivity than cell counting (Scudiero *et al.*, 1988; Roehm *et al.*, 1991; Stevens *et al.*, 1991).

The most common colorimetric assays for determination of cell viability employ tetrazolium salt compounds that are intracellularly converted to coloured formazan products by reducing enzymes present in bacteria or in the mitochondria of eukaryotes in direct proportion to the number of viable cells. The first colorimetric assay developed uses 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Stevens and Olsen, 1993). However, MTT is converted to insoluble purple formazan product by the reducing enzymes of mitochondria, and the purple formazan must then be solubilized in isopropanol prior to measuring the optical density of formazan (Stevens *et al.*, 1991; Stevens and Olsen, 1993). In comparison, the XTT assay employs the tetrazolium compound sodium 3,3'-[1(phenylamino) carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic

acid hydrate (XTT) that is reduced to a water-soluble orange formazan product by live mammalian cells in the presence of an electron coupling reagent like coenzyme Q (Scudiero *et al.*, 1988; Roehm *et al.*, 1991; Stevens and Olsen, 1993). The elimination of the solubilization step allows the optical density of XTT-treated cells to be measured directly from cell culture, which makes the XTT assay quicker and easier to perform than the MTT assay (Stevens and Olsen, 1993).

Although it is clear that the XTT assay is equally effective at measuring cell viability as the MTT assay (Stevens and Olsen, 1993) and that the XTT assay was competent at establishing cell number and proliferation of lymphocytes and tumour cell lines (Scudiero *et al.*, 1988; Roehm *et al.*, 1991), there was some question whether the XTT assay was a reliable means of determining the cell viability of macrophages. To address this, experiments were conducted calibrating the XTT assay to cell number and comparing cell counts to changes in O.D. 450 nm (the XTT assay) of macrophages treated with potassium and calcium channel inhibitors. Thereafter, the effects of potassium channel inhibitor treatment on the cell respiration, as determined using the XTT assay, were assessed over 72 hour incubation periods.

Materials and Methods

XTT Viability Assay

A 50 µL volume of 1 mg/mL sodium 3,3'-[1[(phenylamino)carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate (XTT) (ICN Biomedicals Inc., Aurora, Ont.) and 50 µg/mL 2,3-dimethoxy-5-methyl-1,4-benzoquinone (CoQ) (Sigma, St. Louis, MO) in 1x Dulbecco's phosphate buffered saline (Gibco BRL, Grand Island, NY) were added to each well of the 96-well culture plate (Scuderio *et al.*, 1988; Roehm *et al.*, 1991; Stevens and Olsen, 1993). The plates were gently tapped to mix the contents, incubated for 1 hour at 37 °C and 5% CO_2, and the optical density at 450 nm (O.D. 450 nm) was determined using an automated spectrophotometer (Bio-tek

Instruments Inc., Winooski, VT).

Cell Number Calibration of the XTT Assay

Cells of the P388D.1 and B10-4(S) cell lines seeded into 50 mL polypropylene centrifuge tubes at a density of approximately 1.0 x 10^6 cells/mL were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (HyClone Laboratories Inc., Logan, UT) and 50 µg/mL gentamicin (Gibco BRL, Grand Island, NY) (complete DMEM). The macrophages were allowed to grow in complete DMEM (control) or treated with 25 U/mL IFN-γ and 1 µg/mL LPS, and incubated for 24 hours in suspension cultures. Cells were centrifuged at 1000 x *g* for 10 minutes, and aliquots of each treatment group (DMEM and LPS/IFN-γ) for both cell lines were stained for viability determination using trypan blue and counted using a hemocytometer. Cells were resuspended at a concentration of 2.5 x 10^7 cells/mL in complete DMEM or 25 U/mL IFN-γ/1 µg/mL LPS-supplemented complete DMEM. A serial dilution was prepared from 2.5 x 10^7 cells/mL to 2.5 x 10^4 cells/mL for each treatment group, and three 200 µL aliquots of each dilution was transferred to separate wells of 96-well culture plates. The plates were allowed to adjust to 37 °C and 5% CO_2 for 1 hour, and an XTT assay was performed. The experiments were repeated twice.

Comparison of the Effects of Ion Channel Inhibitor Treatment on the Cell Number and XTT Viability of Macrophages

B10-4(S) and P388D.1 cells were seeded into wells of 96-well culture plates at a density of 1.0 x 10^4 cells/well, allowed to attach for 1 hour at 37 °C and 5% CO_2, treated with 25 U/mL IFN-γ and 1 µg/mL LPS or complete DMEM (control), and simultaneously treated with either the calcium channel inhibitor cobalt chloride (10 µM - 1 mM) or the potassium channel inhibitor tetraethylammonium (100 µM -10 mM). Plates were incubated for 1 hour (0 hour control group), 24 hours, 48 hours, and 72 hours at 37 °C and 5% CO_2

and cells were counted and the XTT assay was performed using appropriate cultures. For each time point and within individual plates, identically treated wells were prepared in triplicate for both cell enumeration and the XTT assay. These experiments were repeated twice.

Cell Counts

The medium was pipetted off of cells and all serum was rinsed away in 300 µL of 1x Dulbecco's phosphate buffered saline (Gibco BRL, Grand Island, NY). Cells were detached from the 96-well culture plate wells by a 10 minute treatment with 75 µL of 0.5% trypsin-0.1 mM EDTA in Milli-Q water. A 75 µL volume of complete DMEM was added to the trypsin-EDTA suspension of cells to inhibit trypsinization, and an equal volume of trypan blue (150 µL) was then added to the well. Cells were stained for 2 minutes and 30 seconds and the number of live and dead cells were enumerated using a hemocytometer.

Effects of Potassium Channel Inhibitors on the XTT-determined Viability of Macrophages

Macrophages of the B10-4(S) and P388D.1 cell lines were seeded into the wells of 96-well tissue culture plates at a density of 1.0×10^4 cells/well and allowed to attach to the bottom of the wells at 37 °C and 5% CO_2 for 1 hour. Macrophages in different culture plates were treated with cobalt chloride (10 µM to 500 µM), tetraethylammonium (100 µM to 10 mM), 4-aminopyridine (10 µM to 2 mM), and quinine (500 nM to 250 µM) in conjunction with complete DMEM (control) or 1 µg/mL LPS and 25 U/mL IFN-γ. Each treatment concentration was done in triplicate, and plates were prepared for the various incubation lengths examined. The macrophage cultures were incubated at 37 °C and 5% CO_2 for 1 hour (0 hour treatment), 24 hours, 48 hours, and 72 hours prior to determining cell respiration through the XTT assay. These experiments were repeated twice.

Statistics

Regression analyses using linear equations and 2nd-order polynomial function

equations were performed on the cell number calibration data using Statview 4.01 software for the PowerMacintosh. Non-parametric Spearman's rank correlations were performed on the comparisons between cell counts and XTT assays using Statview 4.01 software for the Power Macintosh. The data on the effects of potassium channel inhibitors on the XTT viability of macrophages were analyzed using Super-ANOVA software for the Power Macintosh. Differences between experimental groups were determined using one-factor analysis of variance and a least square means test. A BonFerroni correction was applied to all analyses using multiple comparisons, and the p value that was considered to be significant was equal to α/n, where α was the overall desired level of significance for the experiment and n was the number of comparisons. An α less than 0.05 was considered to be statistically significant.

Results

Cell Number Calibration of the XTT Assay

Proportionally, higher number of cells caused an increase in the optical density at 450 nm (O.D. 450 nm) of XTT-CoQ treated cells that could be approximated by a linear curve for resting and 25 U/mL IFN-γ/1 μg/mL LPS activated cells of both the B10-4(S) and P388D.1 cell lines. Nevertheless, the data fit best to a curve of a second order (degree) polynomial function (Figs. A1-1 to A1-4). The curves of the resting and IFN-γ/LPS activated B10-4(S) cells were similer enough that the data could have been combined to produce a single calibration curve (Figs. A1-1 and A1-2); however, the activated P388D.1 cells had a much higher O.D. 450 nm value than the resting P388D.1 cells at a given cell number. This required that separate calibration curves be used for resting and activated P388D.1 cells (Figs. A1-3 and A1-4).

Correlation of Cell Counts to Changes in O.D. 450 nm (XTT Assay)

Spearman rank correlations between the cell number and O.D. 450 nm of resting

and activated B10-4(S) and P388D.1 cells that had been treated with various doses of tetraethylammonium (0 µM, 100 µM, 1 mM, and 5 mM) or cobalt chloride (0 µM, 10 µM, 100 µM, and 500 µM) for different incubation lengths (0 hr, 24 hr, 48 hr, or 72 hr) resulted in significant ($p<0.05$) positive correlations for a vast majority of the treatment groups (Table A1-1). Of the 24 cases examined, 21 cases had p values less than 0.05 and more importantly 16 cases had correlation coefficients (rho values) greater than 0.750. This indicated that a strong positive correlation between relative changes in cell number and relative changes in the O.D. 450 nm values of the XTT assay existed for cells treated with the potassium channel inhibitors.

Effects of Ion Channel Inhibitors on the Cell Respiration of Macrophages

Cobalt chloride

Treating the B10-4(S) cell line with cobalt chloride resulted in a reduction of the cell respiration of resting and IFN-γ/LPS activated cells at the highest cobalt chloride concentrations (Fig. A1-5). Although it would appear that this reduction immediately occurred because of the reduced cell respirations in the 0 hour group, care must be taken in interpreting data from cells that had been given so little time to adjust after manipulation. The unreliability of the 0 hour data is seen in the high standard error of the means (SEM) and large variation from concentration to concentration of the 0 hour data for all of the ion channel inhibitors and not just for cobalt chloride. Nevertheless, the toxicity of the higher concentrations of cobalt chloride was undeniable in the 24 hour group and longer incubations caused further reductions in cell respiration (Fig. A1-5). Similarly for the P388D.1 cell line, the high amounts of cobalt chloride caused immediate (0 hour group) reductions in the cell respiration of resting and IFN-γ/LPS activated cells and caused further decrease in cell respiration after longer incubations (Fig. A1-6). While a concentration of 100 µM cobalt chloride caused a reduction of the O.D. 450 nm of the XTT assay (cell respiration) to half of the 0 µM cobalt chloride control value for the resting and

activated B10-4(S) cells (Fig. A1-5), the activated and resting P388D.1 cells required 100-250 μM cobalt chloride for the XTT viability to be reduced to one-half of control values (Fig. A1-6).

Tetraethylammonium

The cell respiration of the resting B10-4(S) cells treated with TEA was either at control levels or slightly higher for the first 48 hours of incubation (Fig. A1-7A). By 72 hours of incubation, TEA above a 1 mM concentration caused significant reductions in cell respiration (Fig. A1-7A). B10-4(S) cells that had been activated with IFN-γ and LPS reacted sooner to treatment with TEA, showing a significant reduction in cell respiration in response to the 5 mM concentration after 24 hours of incubation (Fig. A1-7B). The cell respiration was further reduced by longer incubations, and lower concentrations of TEA caused significant reductions of cell respiration at longer incubation lengths (Fig. A1-7B). By 72 hours, 1 mM TEA caused a significant reduction in cell respiration of the B10-4(S) cells (Fig. A1-7B). For the P388D.1 cell line, incubation with TEA did not reduce the cell respiration of the resting P388D.1 cells until 48 hours after treatment and of the IFN-γ/LPS activated P388D.1 cells until 72 hours (Fig. A1-8). Only concentrations of TEA above 2.5 mM reduced the cell respiration of the resting P388D.1 cells, and the activated P388D.1 cells required a 5 mM concentration of TEA before there was a significant reduction in cell respiration (Fig. A1-8). The 2.5 mM concentration of TEA produced a 50% reduction in cell respiration of the B10-4(S) cells compared to the control, whereas 5 mM TEA produced a similar reduction in the P388D.1 cells. Unlike the cobalt chloride experiments, the effects of TEA on cell respiration could not be seen until 48 hours of incubation and were not significantly affected until 72 hours of incubation.

4-Aminopyridine (4-AP)

After 24 hours of incubation, treatment with up to 2 mM 4-AP did not significantly affect the cell respiration of either resting or IFN-γ/LPS activated B10-4(S) cells (Fig. A1-

9). The situation was very similar in the P388D.1 cell line except that there were significant reductions in the cell respiration at 24 hours post-treatment (Fig. A1-10). The resting cells experienced a significant reduction at 2 mM 4-AP, whereas the intermediate concentrations of 4-AP (50 µM - 1 mM) caused significant reductions in the cell respiration of IFN-γ/LPS activated P388D.1 cells (Fig. A1-10). By 48 hours of incubation these slight reductions in cell respiration had recovered, and the cell respiration of the resting and IFN-γ/LPS activated P388D.1 cells remained at or above control levels (Fig. A1-10).

Quinine

Only the highest concentrations (50-250 µM) of quinine significantly reduced the cell respiration of either the resting or the IFN-γ/LPS activated B10-4 cells (Fig. A1-11). These reductions were seen by 24 hours post-treatment, but longer incubations did not cause further reductions in cell respiration in the B10-4 cells (Fig. A1-11). Concentrations of quinine above 100 µM significantly reduced the cell respiration of both the resting and the IFN-γ/LPS activated P388D.1 cells after 24 hours of incubation (Fig. A1-12). Longer incubations caused further reductions in the cell respiration of resting and activated P388D.1 cells and by 72 hours post-treatment, 50 µM quinine caused a significant reduction in the cell respiration (Fig. A1-12).

Discussion

The better fit of the 2nd-degree polynomial curves to the cell number calibration data, as seen by the higher R-squared values, shows that the change in O.D. 450 nm from the XTT assay with changing numbers of cells is not a linear relationship. Rather, it appears that changes in O.D. 450 nm caused by increasing numbers of cells is asymptotic at higher cell densities with a unit increase in cell number producing increasingly smaller changes in the O.D. 450 nm of the XTT assay (Figs. A1-1 to A1-4). Nevertheless, for each calibration treatment there existed a range of cell numbers where the relationship

between cell number and O.D. 450 nm could be approximated by a linear curve. At low cell concentrations (usually below 1.0 x 10^4 cells/well) or high cell concentrations (usually above 5.0 x 10^5 cells/well), the O.D. 450 nm values deviated from the linear portion of the calibration curve (Figs. A1-1 to A1-4). To accomodate this limitation of the XTT assay, all the experiments were conducted using cell numbers between 1.0 x 10^4 cells/well and 2.5 x 10^5 cells/well. A seeding density of 1.0 x 10^5 cells/well was most commonly used since its O.D. 450 nm value was in the middle of the linear portion of the calibration curve.

Examining the effects of potassium channel inhibitors on both cell number and the O.D. 450 nm from the XTT assay of B10-4(S) cells and P388D.1 cells treated with TEA and cobalt chloride confirmed that the XTT assay was a reasonable relative measure of cell viability. There was clearly a correlation between changes in cell number and changes in the O.D. 450 nm of the XTT assay. In general, when there was an increase or decrease in cell number for a particular treatment, there was a similar change in the O.D. 450 nm for the same treatment. Of the 8 cases that had rho coefficients below 0.750, all but two were using P388D.1 or B10-4(S) cells that had been activated with 25 U/mL IFN-γ and 1 μg/mL LPS. Rather than reflecting that there was truly a reduced correlation between cell number and O.D. 450 nm for activated cells, the reduced rho coefficients likely reflect the increased difficulty experienced in detaching the activated cells from the wells for cell counting. Activation of macrophages causes the cells to upregulate expression of a number of cell adhesion and extracellular matrix proteins such as CAM-1 and fibronectin in preparation for migration and phagocytosis (Abbas *et al.*, 1994). This increases the macrophages ability to attach to and spread over surfaces compared to resting macrophages. The increased number of attachment proteins and the greater number of contacts with the substrate by the spreading cells likely required even harsher or longer treatments with trypsin-EDTA to detach the activated macrophages as effectively as the resting cells were detached. In some experimental trials, either almost no cells were

counted or the cell number did not increase with longer incubation lengths even though cells could be seen under inverted phase contrast microscopy to be near confluency or perceptibly increasing in density with time. These observations suggest that problems with experimental design rather than an actual lack of correlation were responsible for the lower rho values of the activated macrophage trials. Even so, significant positive correlations ($p<0.05$) between cell number and O.D. 450 nm were still observed in 8 of 12 trials using activated macrophages. Although the degree of correlation was reduced (lower rho values) there was clearly still a correlation between cell number and O.D. 450 nm values of the XTT assay for activated macrophages. Therefore, I believe the O.D. 450 nm from the XTT assay can be used to measure relative changes in cell viability caused by potassium channel inhibitors.

The O.D. 450 nm value for a particular cell density and treatment was usually consistent within a particular trial, but there were some differences between trials. This may have been because of differences in the exact seeding densities used between trials, possible differences in the quality of the light-sensitive reagents (XTT and CoQ), or because of differences in the rate of metabolism for macrophages used in different trials. These differences in O.D. 450 nm values made combining or comparing the O.D. 450 nm data between trials difficult, if not impossible, but did not affect the usefulness of the XTT assay for measuring the effects of potassium channel inhibitor treatments on the cell viability within a single trial. For this reason, control cultures were included on the same plate as the ion channel inhibitor treatments so that comparisons could be made (the control cultures would be seeded with the same cells as the treatment groups and would be exposed to the same quality of XTT and CoQ). Regardless of whether the exact O.D. 450 nm values for particular treatments varied between trials, the overall trends seen in the various treatments using the XTT assay were consistent. As such, the XTT assay was used as a relative measure of viability compared to a control within a single trial to ensure that

concentrations of ion channel inhibitors were being used that did not affect cell viability.

The data from the experiments assessing the effects of ion channel inhibitors on macrophage viability indicated which concentrations of the ion channel inhibitors would minimally affect the viability of the cell lines. Concentrations of the ion channel inhibitors that caused less than a 50% reduction in XTT viability at 24 hours of incubation were used for further experiments so that the actual effects of the potassium channel inhibitors on cellular metabolism relating to cytotoxic activation could be examined.

Table A1-1. Results of Spearman rank correlation tests between cell number and O.D. 450 nm values of resting and 1 μg/mL LPS/25 U/mL IFN-γ activated B10-4(S) and P388D.1 cells treated with ion channel inhibitors.

Cell Line	Treatment	Trial	Rho	p-Value
Rest. B10-4(S)	Cobalt chloride	1	0.815	0.0016
		2	0.873	0.0007
		3	0.933	0.0003
	TEA	1	0.786	0.0023
		2	0.624	0.0157
		3	0.853	0.0010
Act. B10-4(S)	Cobalt chloride	1	0.226	0.3820
		2	0.786	0.0023
		3	0.827	0.0014
	TEA	1	-0.010	0.9682
		2	0.882	0.0006
		3	0.501	0.0525
Rest. P388D.1	Cobalt chloride	1	0.918	0.0004
		2	0.801	0.0019
		3	0.979	0.0012
	TEA	1	0.929	0.0003
		2	0.823	0.0014
		3	0.855	0.0009
Act. P388D.1	Cobalt chloride	1	0.801	0.0019
		2	0.546	0.0344
		3	0.972	0.0013
	TEA	1	-0.814	0.0016
		2	0.650	0.0118
		3	0.635	0.0139

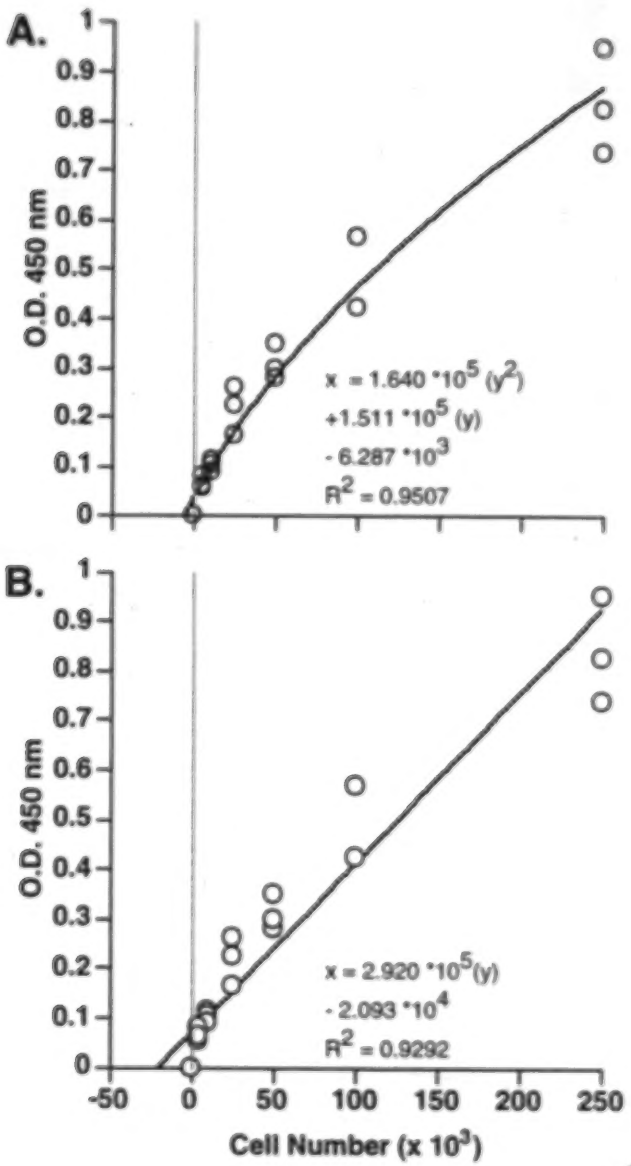

Figure A1-1. Cell number calibration of the XTT assay for resting B10-4(S) cells. The relationship between the number of resting B10-4(S) cells and the change in O.D. 450 nm following the XTT assay as a second-degree polynomial regression (A.) and a linear regression (B.) was examined.

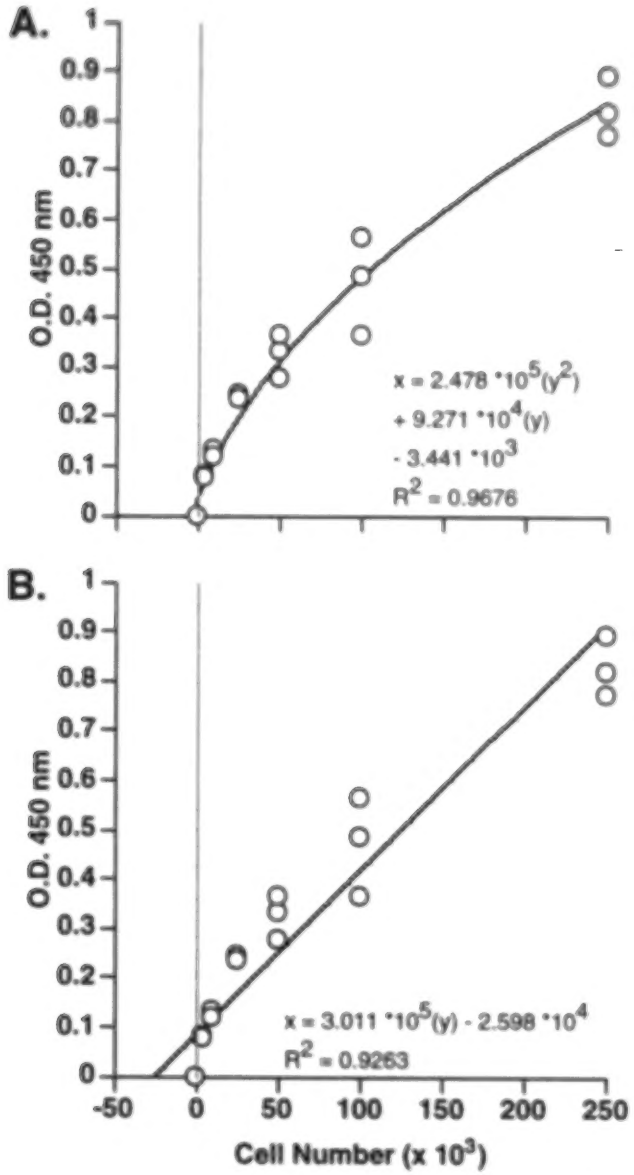

Figure A1-2. Cell number calibration of the XTT assay for activated B10-4(S) cells. The relationship between the number of 1 μg/mL LPS/25 U/mL IFN-γ activated B10-4(S) cells and the change in O.D. 450 nm following the XTT assay as a second-degree polynomial regression (A.) and a linear regression (B.) was examined.

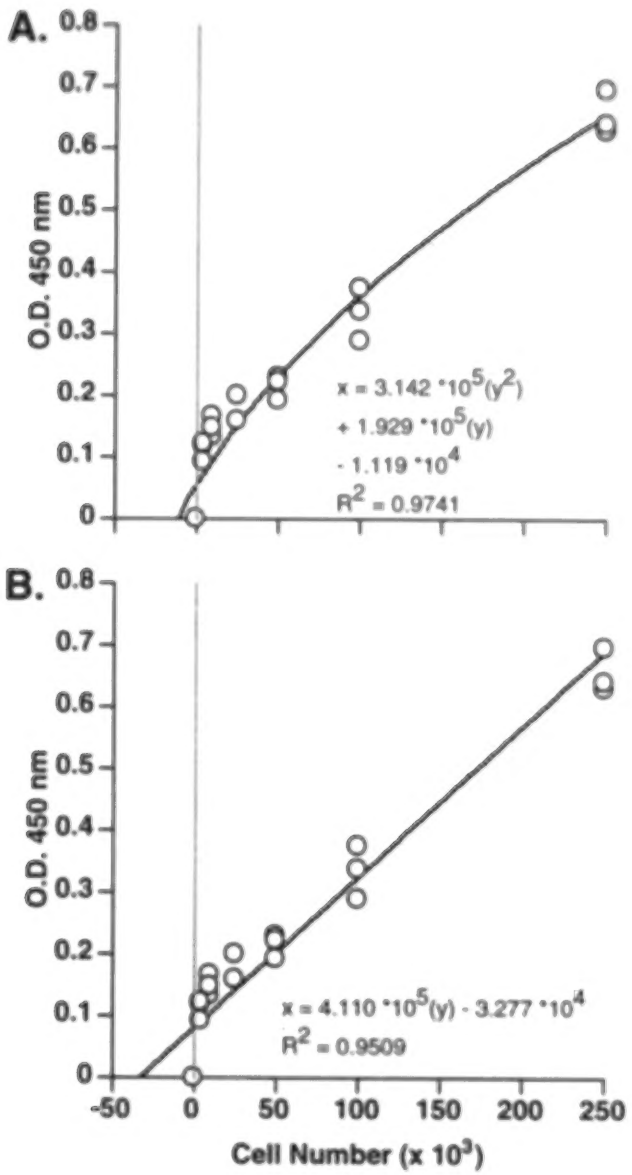

Figure A1-3. Cell number calibration of the XTT assay for resting P388D.1 cells. The relationship between the number of resting P388D.1 cells and the change in O.D. 450 nm following the XTT assay as a second-degree polynomial regression (A.) and a linear regression (B.) was examined.

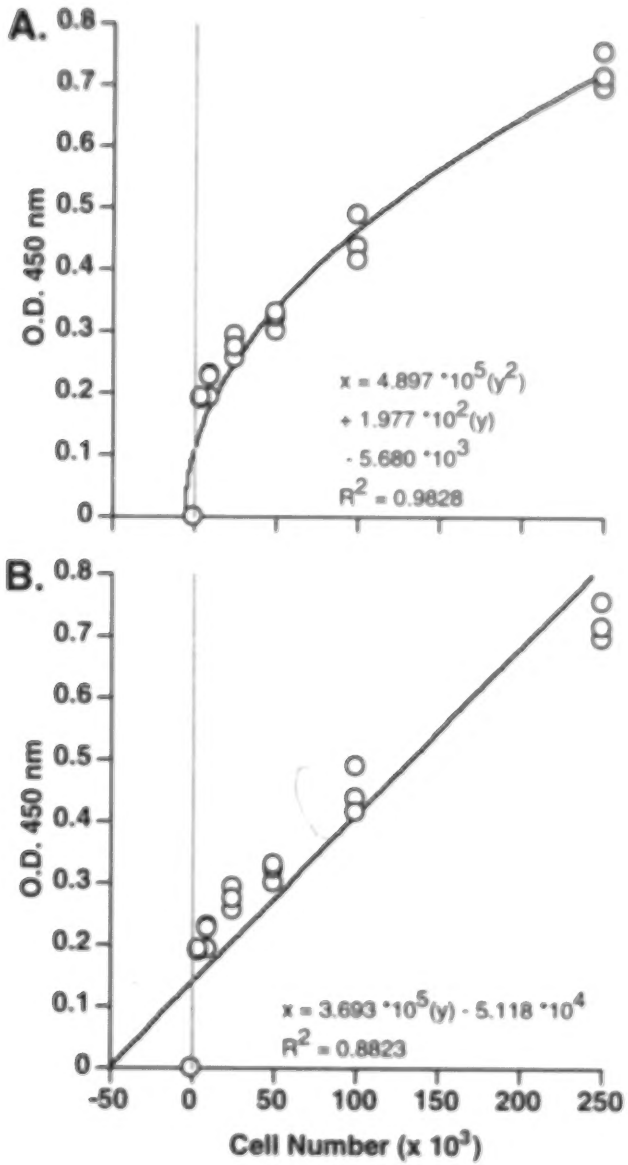

Figure A1-4. Cell number calibration of the XTT assay for activated P388D.1 cells. The relationship between the number of 1 μg/mL LPS/25 U/mL IFN-γ activated P388D.1 cells and the change in O.D. 450 nm following the XTT assay as a second-degree polynomial regression (A.) and a linear regression (B.) was examined.

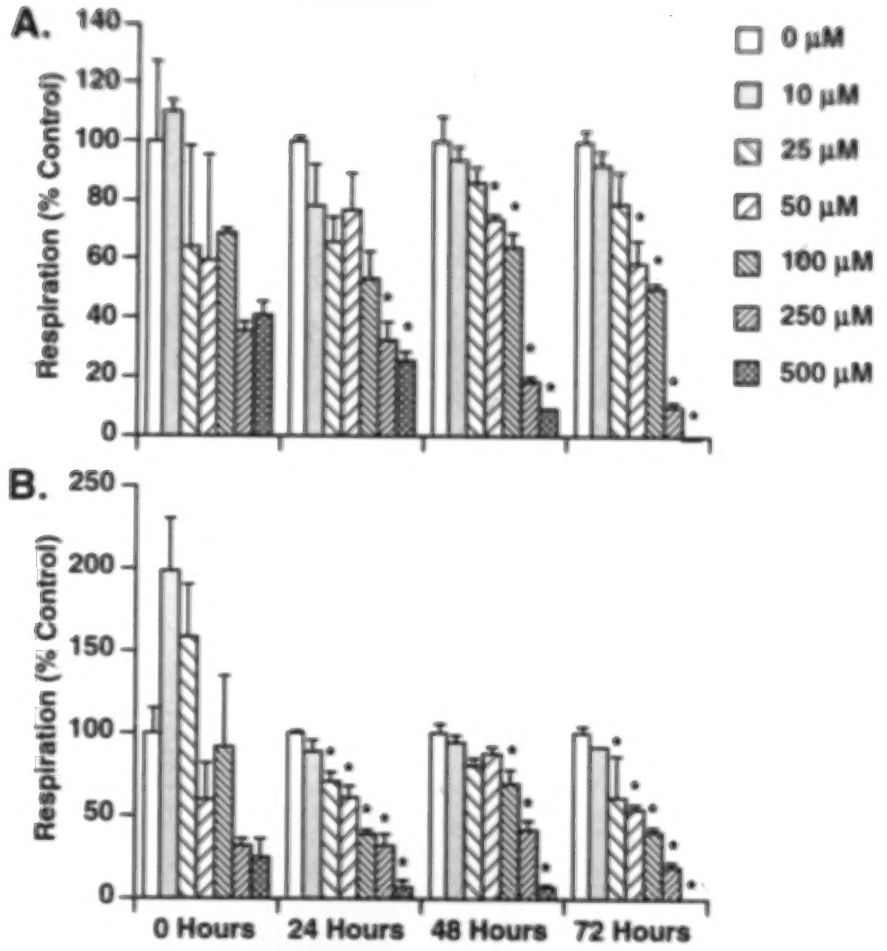

Figure A1-5. Cobalt chloride on B10-4(S) Viability. The effects of several cobalt chloride concentrations on the cell viability of resting (A.) and 25 U/mL IFN-γ/1 µg/mL LPS activated (B.) B10-4(S) cells incubated for up to 72 hours. Control O.D. 450 nm values (Resting±SEM /Activated±SEM): 0 Hr - (0.042±0.011/0.028±0.004); 24 Hr - (0.195 ±0.003/0.344±0.002); 48 Hr - (0.375±0.032/0.434 ±0.024); 72 Hr - (0.569±0.024/ 0.516±0.018). A * indicates a significant difference ($p<0.05$) from the 0 µM control.

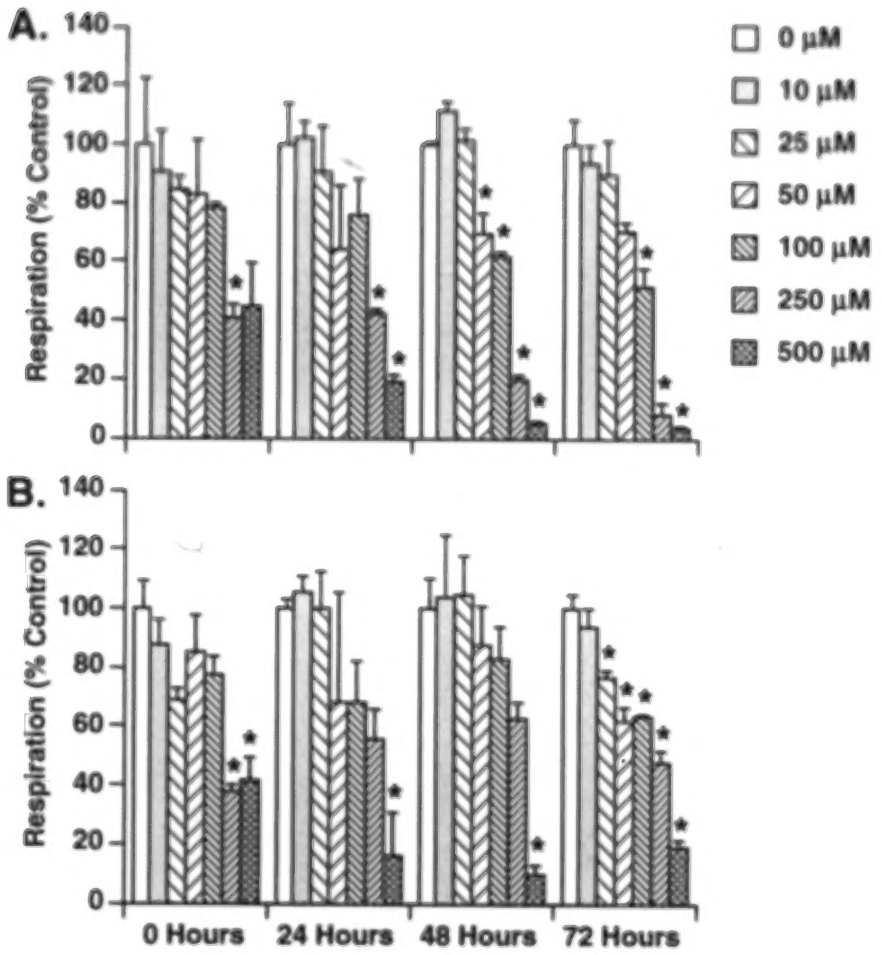

Figure A1-6. Cobalt chloride on P388D.1 Viability. The effects of several cobalt chloride concentrations on the cell viability of resting (A.) and 25 U/mL IFN-γ/1 µg/mL LPS activated (B.) P388D.1 cells incubated for up to 72 hours. Control O.D. 450 nm values (Resting±SEM /Activated±SEM): 0 Hr - (0.052±0.012/0.044±0.004); 24 Hr - (0.090 ±0.013/0.127±0.004); 48 Hr - (0.257±0.002/0.273 ±0.027); 72 Hr - (0.358±0.030/ 0.355±0.018). A * indicates a significant difference ($p<0.05$) from the 0 µM control.

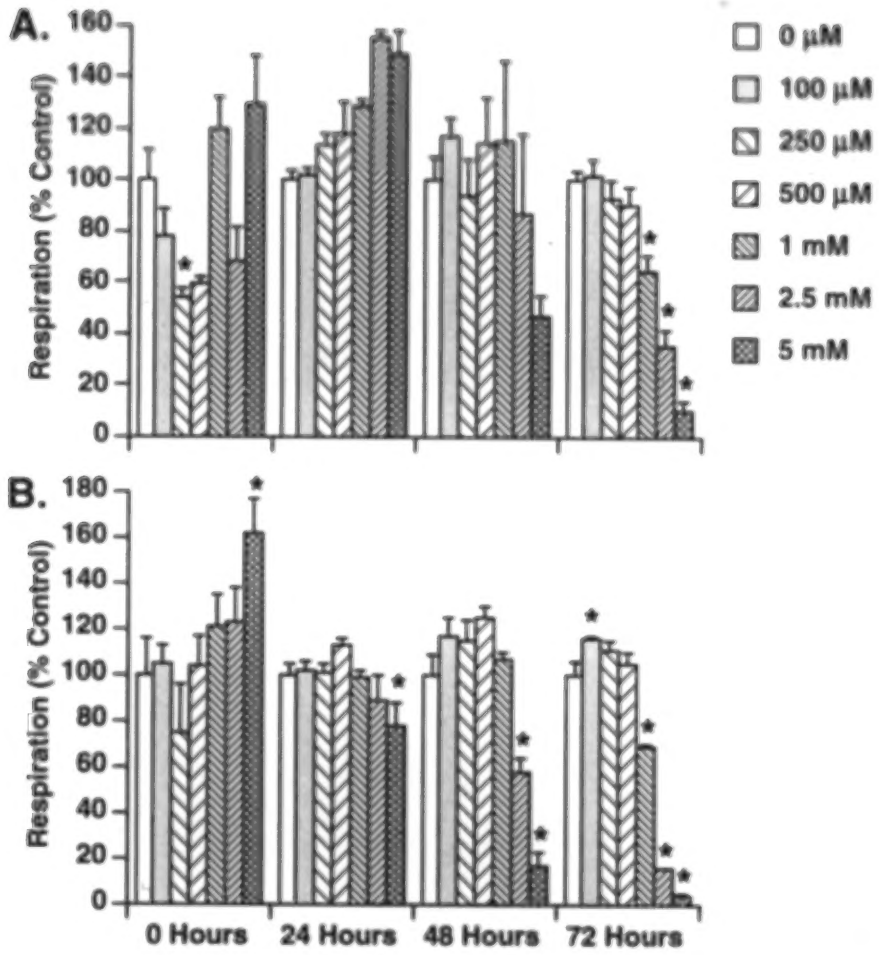

Figure A1-7. Tetraethylammonium on B10-4(S) Viability. The effects of several tetraethylammonium concentrations on the cell viability of resting (A.) and 25 U/mL IFN-γ /1 µg/mL LPS activated (B.) B10-4(S) cells incubated for up to 72 hours. Control O.D. 450 nm values (Resting±SEM /Activated±SEM): 0 Hr - (0.056±0.006/0.035±0.005); 24 Hr - (0.128 ±0.004/0.235±0.011); 48 Hr - (0.328±0.029/0.361 ±0.032); 72 Hr - (0.643±0.023/0.655±0.035). A * indicates a significant difference ($p<0.05$) from the 0 µM control.

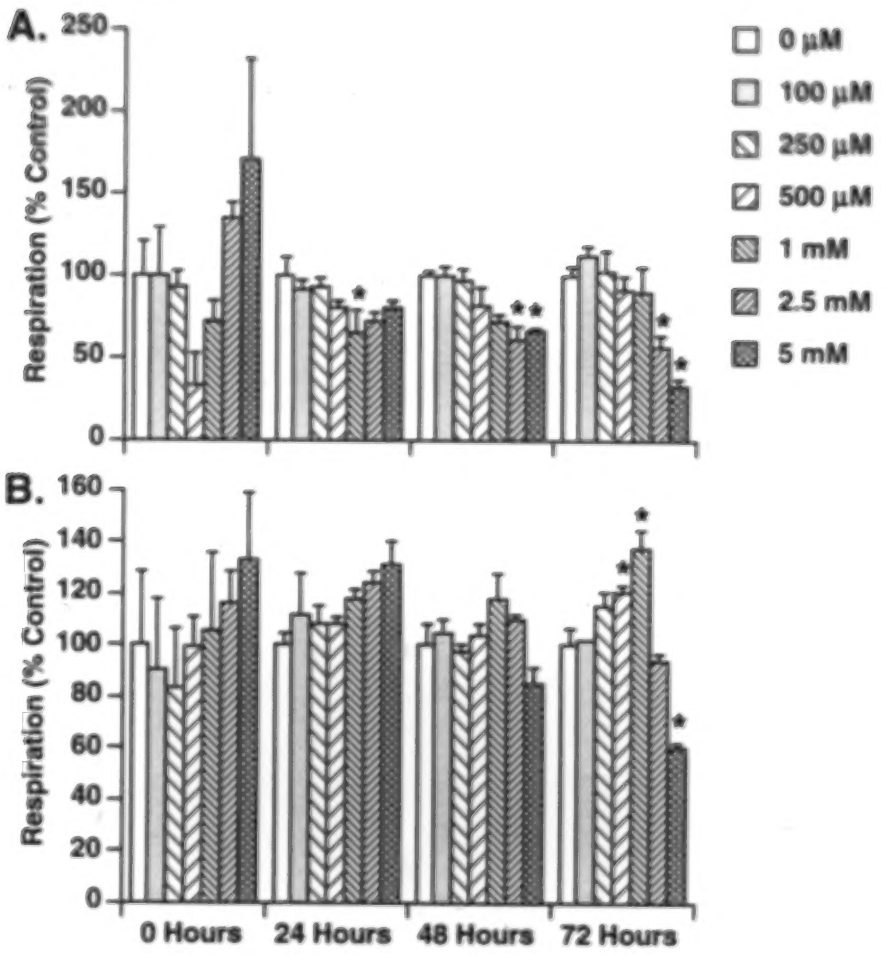

Figure A1-8. Tetraethylammonium on P388D.1 Viability. The effects of several tetraethylammonium concentrations on the cell viability of resting (A.) and 25 U/mL IFN-γ /1 µg/mL LPS activated (B.) P388D.1 cells incubated for up to 72 hours. Control O.D. 450 nm values (Resting±SEM /Activated±SEM): 0 Hr - (0.030±0.006/0.031±0.009); 24 Hr - (0.096 ±0.011/0.177±0.007); 48 Hr - (0.199±0.005/0.296 ±0.023); 72 Hr - (0.492±0.023/0.404±0.027). A * indicates a significant difference ($p<0.05$) from the 0 µM control.

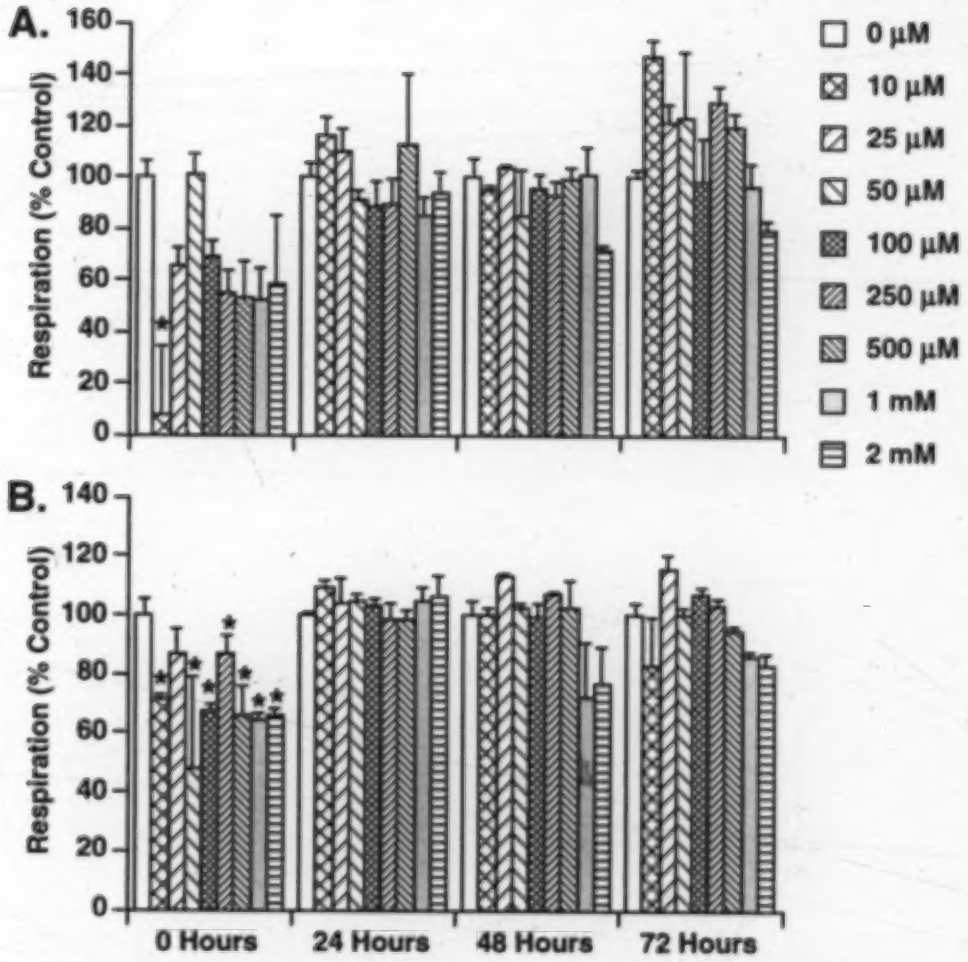

Figure A1-9. 4-aminopyridine on B10-4(S) Viability. The effects of several 4-aminopyridine concentrations on the cell viability of resting (A.) and 25 U/mL IFN-γ/1 µg/mL LPS activated (B.) B10-4(S) cells incubated for up to 72 hours. Control O.D. 450 nm values (Resting±SEM /Activated±SEM): 0 Hr - (0.069±0.004/0.062±0.004); 24 Hr - (0.204 ±0.011/0.255±0.002); 48 Hr - (0.392±0.027/0.323 ±0.016); 72 Hr - (0.453± 0.012/0.410±0.016). A * indicates a significant difference ($p<0.05$) from the 0 µM control.

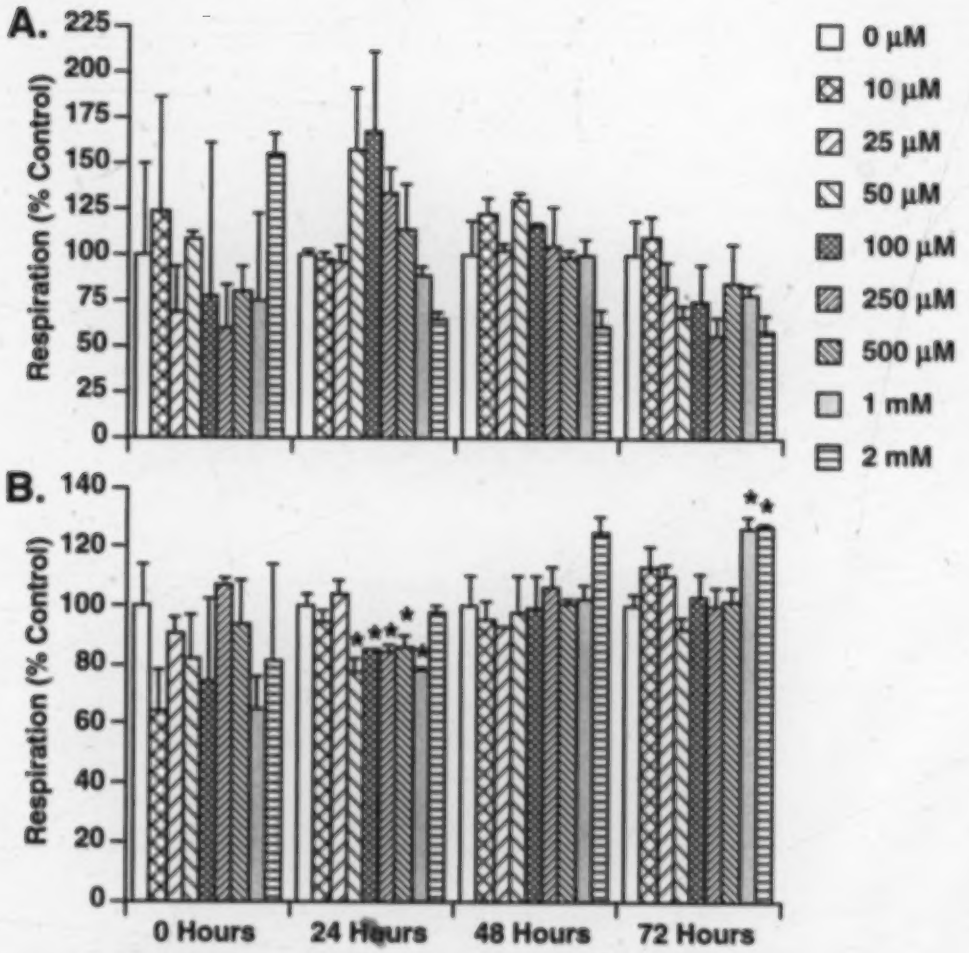

Figure A1-10. 4-aminopyridine on P388D.1 Viability. The effects of several 4-aminopyridine concentrations on the cell viability of resting (A.) and 25 U/mL IFN-γ/1 µg/mL LPS activated (B.) P388D.1 cells incubated for up to 72 hours. Control O.D. 450 nm values (Resting±SEM /Activated±SEM): 0 Hr - (0.012±0.006/0.043±0.006); 24 Hr - (0.054 ±0.001/0.219±0.009); 48 Hr - (0.177±0.032/0.228 ±0.023); 72 Hr - (0.202± 0.038/0.316±0.013). A * indicates a significant difference ($p<0.05$) from the 0 µM control.

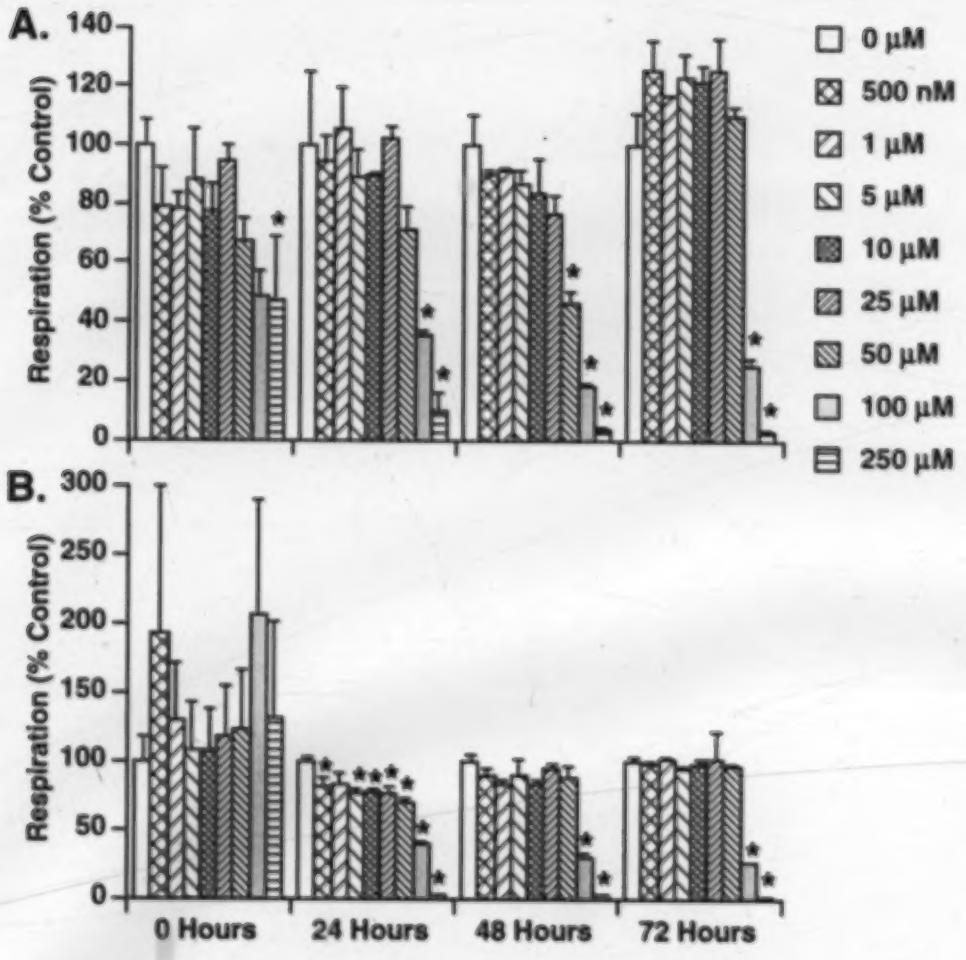

Figure A1-11. Quinine on B10-4(S) Viability. The effects of several quinine concentrations on the cell viability of resting (A.) and 25 U/mL IFN-γ/1 µg/mL LPS activated (B.) B10-4(S) cells incubated for up to 72 hours. Control O.D. 450 nm values (Resting±SEM /Activated±SEM): 0 Hr - (0.059 ±0.005/0.020±0.004); 24 Hr - (0.173 ±0.044/0.353± 0.011); 48 Hr - (0.338±0.034/0.545 ±0.024); 72 Hr - (0.386±0.042 /0.822±0.016). A * indicates a significant difference ($p<0.05$) from the 0 µM control.

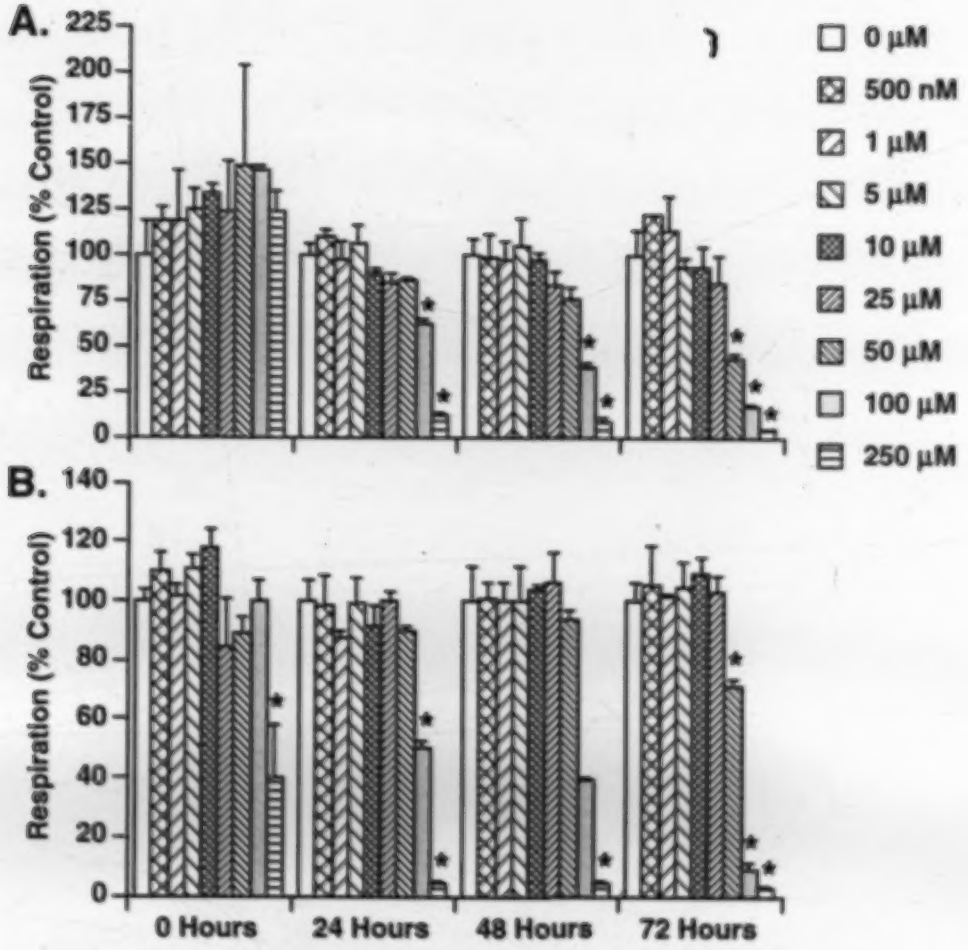

Figure A1-12. Quinine on P388D.1 Viability. The effects of several quinine concentrations on the cell viability of resting (A.) and 25 U/mL IFN-γ/1 µg/mL LPS activated (B.) P388D.1 cells incubated for up to 72 hours. Control O.D. 450 nm values (Resting±SEM /Activated±SEM): 0 Hr - (0.026 ±0.005/0.040±0.002); 24 Hr - (0.096±0.006/0.189 ±0.014); 48 Hr - (0.162±0.014/0.214 ±0.026); 72 Hr - (0.281±0.038 /0.308±0.020). A * indicates a significant difference ($p<0.05$) from the 0 µM control.